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# A Rapid and Simple HPLC Method for Therapeutic Monitoring of Vancomycin

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## Abstract

Therapeutic monitoring of the antibiotic vancomycin is important to achieve specific plasma concentration and prevent toxic effects. Several assays have been described for vancomycin determination in clinical practice, but high-performance liquid chromatography is still considered the gold standard for the quantification of vancomycin. In this study, we developed a new and rapid high-performance liquid chromatography method requiring 50 µL of plasma for the quantification of vancomycin. Acetonitrile was used for processing plasma by protein precipitation (1:2.5). Isocratic chromatographic analysis was carried out on a C<sub>18</sub> silica-based (2.7 µm) column with the mobile phase containing 20 mM ammonium acetate/formic acid buffer (pH 4.0):methanol 88:12 (v/v). A diode array detector was used for UV detection at 240 nm. This method was validated according to the Brazilian Health Surveillance Agency legislation and International Conference on Harmonization guidelines. The measurement range was 1–100 µg/mL, analysis time was 8 min, and intermediate precision was <12%, supporting the present method as a fast, simple, and effective alternative for therapeutic monitoring of vancomycin.

## Introduction

Vancomycin, a glycosylated peptide antibiotic obtained from *Amycolatopsis orientalis*, was approved and introduced in clinical practice in 1958 for the management of aerobic gram-positive infections. The use of vancomycin was overshadowed by the development of beta-lactams, particularly methicillin and cephalothin (1–3). The worldwide development of bacterial resistance to these drugs and the increase in methicillin-resistant *Staphylococcus aureus* (MRSA) in the 1980s played a role in the resurgence of vancomycin use (4). Despite the emergence of new and alternative drugs

(linezolid, daptomycin and tigecycline), vancomycin remains the drug of choice for the treatment of severe MRSA infections (5).

Therapeutic monitoring of vancomycin was first proposed to prevent toxic effects related to the therapy, such as ototoxicity in elderly patients and nephrotoxicity in high-dose therapy (6–9). In recent years, however, monitoring vancomycin levels is mostly used to achieve specific plasma concentration for therapeutic success.

A consensus statement from the American Society of Health-System Pharmacists, the Infectious Diseases Society of America, and the Society

of Infectious Diseases Pharmacists recommends that the trough vancomycin plasma level should be in the range of 15–20 µg/mL for severe infections, and >10 µg/mL to prevent resistance development (10).

Several techniques have been described for the determination of vancomycin, including bioassay, high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), fluorescence polarization immunoassay (FPIA), and enzyme multiplied immunoassay technique (EMIT) (11–14). Immune enzymatic techniques, especially FPIA, are used clinically for the therapeutic monitoring of vancomycin due to their feasibility and rapid analysis (15–18). However, HPLC is more sensitive and specific, and suitable for the detection of lower vancomycin levels with high accuracy and precision. Moreover, HPLC methods, post the initial equipment investment, are more cost effective compared to other methods (19–21).

The present study aims at developing a new and rapid HPLC method with UV detection for the vancomycin assay in human plasma.

## Experimental

### Materials and reagents

The reference standard vancomycin was purchased from Sigma Aldrich (São Paulo, Brazil). The internal standard (IS) Zidovudine was supplied by Cristália Pharmaceutical Industry (São Paulo, Brazil). Acetonitrile, methanol, ammonium acetate, and formic acid were obtained from Tedia Brasil. Ultrapure water was obtained from Elga USFilter system (Garden Grove, CA, USA). Blank human plasma was obtained from the blood bank of HEMORIO (Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti).

### Instrumentation and chromatographic conditions

An Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) with diode array detector (DAD) equipped with Chemstation software package was used for method development and validation. Chromatographic separation was performed on a Supelcosil C<sub>18</sub> column (150 × 4.6 mm, 2.7 µm particle size, 90 Å pore size, Sigma Aldrich), maintained at 50°C. Ultraviolet measurements were carried out at 240 nm. The mobile phase was 20 mM ammonium acetate/formic acid buffer (pH 4.0): methanol 88:12 (v/v) with a flow rate of 1.5 mL/min. The sample injection volume was 20 µL, and the running time for each sample was 8 min.

### Preparation of stock solutions, calibration curve and quality control samples

Stock vancomycin solutions were prepared by dissolving 10 mg of vancomycin in 10 mL of ultrapure water (1,000 µg/mL). Dilutions with ultrapure water were used for preparing standard vancomycin solutions (1–100 µg/mL). The internal standard stock solution was prepared by dissolving 10 mg of Zidovudine in 10 mL of ultrapure water, and the internal standard working solution (10 µg/mL) was obtained by diluting the stock solution with acetonitrile.

Calibration samples were established by diluting the aforementioned solutions with blank human plasma to final concentrations of 1.0, 3.0, 10.0, 20.0, 50.0, 70.0 and 100.0 µg/mL. The limit of quantification (LoQ) sample was prepared separately at a concentration of 1.0 µg/mL. For vancomycin quality control samples, the low quality control (LQC), medium quality control (MQC), high quality control (HQC), and dilution quality control (DQC) were prepared separately at four concentration levels of 5.0, 40.0, 80.0 and 200.0 µg/mL, respectively. All the samples were stored at –20°C.

### Sample extraction

A 50 µL aliquot of standard or quality control sample, followed by 125 µL of IS working solution, was added to a microtube. The sample was vortexed for 30 s and centrifuged at 10,000 rpm with maximum relative centrifugation force (RCF) of 10 956 × g for 10 min at room temperature. The supernatant was placed in a glass tube and dried in a vacuum sample concentrator for 40 min at 60°C. The residue was reconstituted with 50 µL of ammonium acetate buffer pH 4 and placed in an auto sampler vial for injection.

### Method validation

Method validation was performed in accordance with the Brazilian Health Surveillance Agency, RDC n° 27/2012 (22) and International Conference on Harmonization (ICH) guidelines (23). The following parameters were considered: specificity, matrix effect, carry-over, linearity, precision, accuracy and drug stability in plasma and solution.

Specificity was validated by injecting six blank plasma samples (four with normal plasma, one with hemolyzed plasma, and one with lipemic plasma) obtained from individual donors, and the results were compared to the LoQ and IS plasma samples in order to identify possible interferences from other components on the chromatographic peaks of the analyte and/or IS.

For assessing the matrix effects, eight blank plasma samples (four with normal plasma, two with hemolyzed plasma, and two with lipemic plasma) obtained from individual donors were first processed and further spiked with vancomycin and IS to achieve the LQC and HQC levels. The spiked samples were compared to the analytes in solution at the same levels. The IS-normalized MF was calculated for each sample, and the results were expressed in terms of coefficient of variation (CV%).

Carry-over was evaluated by the injection of blank plasma sample in triplicate, one before and two after the injection of spiked plasma sample at 100.0 µg/mL vancomycin.

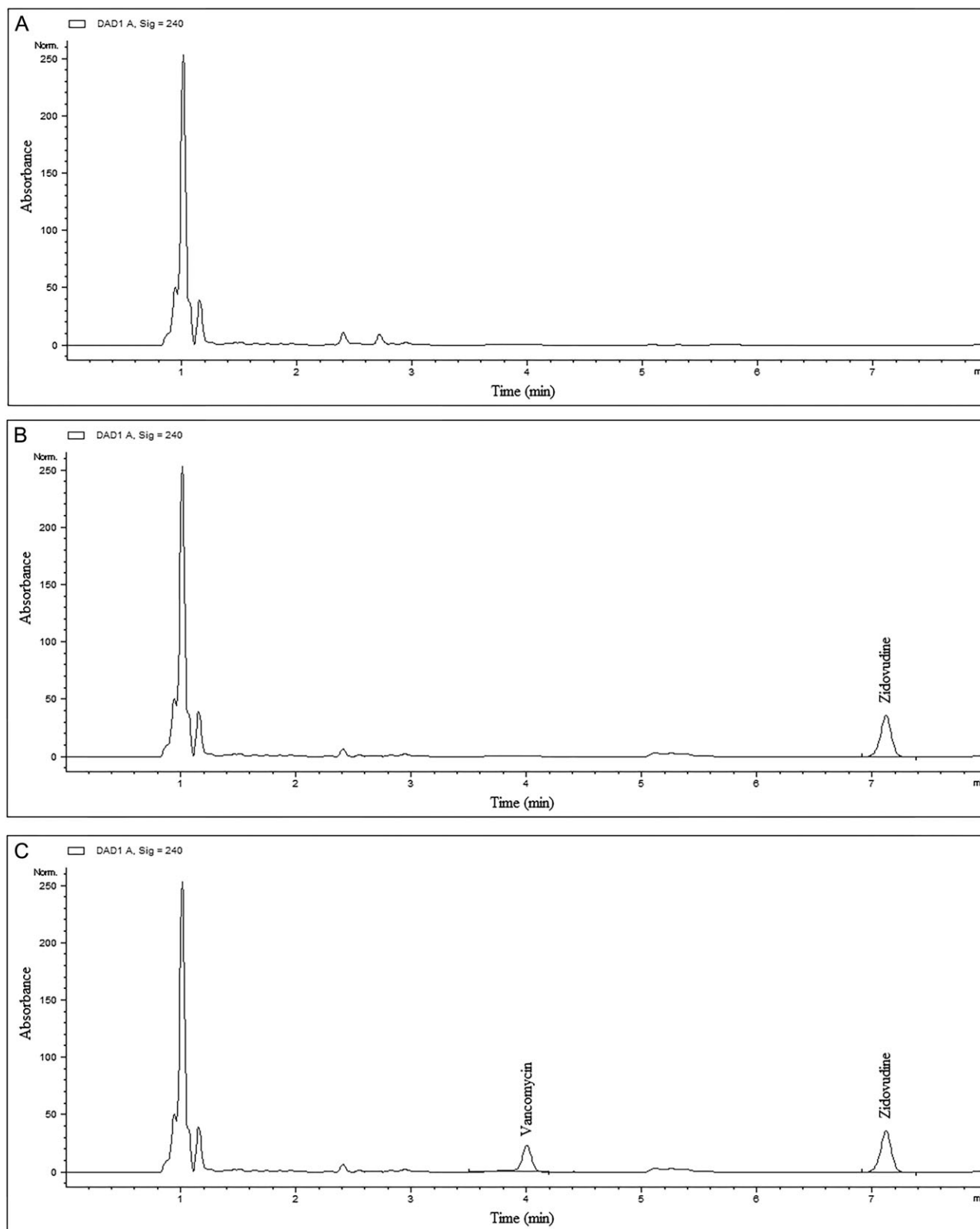
Linearity was achieved by injection of seven calibration samples in triplicate, covering a calibration curve range of 1.0–100.0 mg/mL vancomycin. The calibration curve was obtained by least-squares linear regression analysis.

The precision of the method was determined as repeatability precision and intermediate precision. Repeatability was determined by analyzing seven replicate injections of five preparations (LoQ, LQC, MQC, HQC and DQC) on the same day. For the intermediate precision study, analysis was performed by injecting four preparations (LoQ, LQC, MQC, HQC) in seven replicates on three consecutive days. The results were expressed in terms of CV%.

Accuracy was assessed as within-run and between-run accuracy. Tests for within-run accuracy and between-run accuracy were performed in a manner similar to the repeatability precision and intermediate precision tests, respectively. Accuracy was expressed in terms of recovery (%).

Samples containing vancomycin at 10.0 and 100.0 µg/mL (in triplicate) were evaluated for assessing vancomycin and IS stability in solution, on the first and last day of method validation by comparing both the chromatographic peak areas.

The stability of vancomycin in plasma was evaluated using low and high QC sample concentrations (in triplicate) for freeze–thaw cycles as well as short-term and post-preparative stabilities; and by using 10.0 and 20.0 µg/mL vancomycin concentration samples (in triplicate) for long-term stability. The freeze–thaw stability of vancomycin was determined over three cycles within 3 days. In each cycle,



**Figure 1.** Representative HPLC chromatograms of blank human plasma (A), blank human plasma spiked with 50 µg/mL of zidovudine (IS) (B), and blank human plasma spiked with 50 µg/mL of vancomycin and 50 µg/mL of zidovudine (IS) (C).

low and high QC samples were stored at  $-20^{\circ}\text{C}$  for 24 h and thawed at room temperature ( $22\text{--}25^{\circ}\text{C}$ ). When completely thawed, the samples were refrozen for 24 h at  $-20^{\circ}\text{C}$ . For the post-preparative and short-term stabilities, the plasma samples were maintained at room temperature for 12 and 24 h, respectively, and then analyzed. The long-term stability was evaluated after freezing the plasma samples at  $-20^{\circ}\text{C}$  for 14 months.

### Assay applicability

To evaluate the applicability of the method, plasma samples of patients who used vancomycin were collected from the University Hospital Clementino Fraga Filho in Rio de Janeiro. The study was approved by the University Hospital Clementino Fraga Filho Research Ethics Committees for humans (n° 811/11).

The samples were collected 30 min immediately before the second, fourth, or fifth dose administration of vancomycin in the steady

state. Blood samples (10.0 mL) were collected in tubes containing sodium EDTA. Subsequently, plasma was obtained by sample centrifugation at 30,000 rpm with a maximum RCF of  $98,608 \times g$  for 30 min at room temperature. All samples were stored at  $-20^{\circ}\text{C}$  up to analysis.

## Results

In summary, all validation acceptance requirements of the Brazilian Health Surveillance Agency and ICH guidelines were fulfilled.

### Selectivity

No significant interfering peaks were observed in the blank plasma samples at the retention times of vancomycin and the IS. The peaks of vancomycin and IS were observed at retention times of 4.0 and 7.1 min, respectively. The chromatograms of human blank plasma,

**Table I.** Precision and Accuracy Data

Nominal concentration ( $\mu\text{g/mL}$ )	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	Repeatability precision (CV%)	Within-run accuracy (Recovery %)	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	Intermediate precision (CV%)	Between-run accuracy (Recovery %)
1	1.0	6.8	95.4	1.0	8.3	103.3
5	4.9	5.6	105.8	4.8	11.4	99.3
40	40.9	7.4	100.6	39.9	2.5	102.4
80	80.9	5.4	100.5	80.7	3.5	102.3
200	219.0	1.9	109.5	-	-	-

(-) Not evaluated.

<sup>a</sup>The calculated concentrations are reported as mean.

**Table II.** Stability Data of Vancomycin in Plasma

Nominal concentration ( $\mu\text{g/mL}$ )	Post-preparative		Short-term		Freeze-thaw		Long-term	
	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	DV (%)	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	DV (%)	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	DV (%)	Obtained concentration ( $\mu\text{g/mL}$ ) <sup>a</sup>	DV (%)
5	5.17	3.53	5.17	3.50	4.67	6.50	-	-
10	-	-	-	-	-	-	8.81	11.80
20	-	-	-	-	-	-	19.07	4.63
80	81.57	1.96	82.61	3.26	78.29	2.12	-	-

(-) Not evaluated.

<sup>a</sup>The determined concentrations are reported as mean. DV, Deviation compared to nominal concentration.

**Table III.** Clinical and Laboratory Characteristics of the Patients

Patient	1	2	3	4	5	6	7
Sex	M	M	M	M	M	F	M
Age (years)	48	61	59	77	30	79	55
Weight (kg)	52	55	70	50	65	60	52
ECC (mL/min)	83	40	78	<10	76	43	<10
Loading dose (mg)	1,500	1,500	1,500	1,500	1,500	1,500	1,500
Maintenance dose (mg)	1,250	500	1,250	1,000	1,000	500	1,000
Dose interval (h)	12	12	12	96	12	12	96
Collection time (h)	48	48	48	96	48	60	96
Calculated concentration (mg/L) <sup>a</sup>	22.51	14.23	18.36	5.77	13.05	8.68	7.47

M, male; F, female; ECC, Estimated Creatinine Clearance.

<sup>a</sup>Mean of obtained concentrations (triplicate).

human blank plasma spiked with 10.0 µg/mL IS, and human blank plasma spiked with 50.0 µg/mL vancomycin and 50.0 µg/mL IS are shown in Figure 1A–C, respectively.

### Carry-over and matrix effects

No carry-over from previous analyses was observed. For matrix effects, the CV% of relative NMF for all samples (LQC and HQC) ranged from 9.8% to 11.4%.

### Linearity and lower limit of quantification

The calibration curves were linear in the concentration range of 1.0–100.0 µg/mL, with a coefficient of determination ( $R^2$ ) > 0.998, indicating good linearity. No deviation >13% for LoQ and >8% for other QC levels was observed. The LoQ was 1 µg/mL.

### Precision and accuracy

Table I shows the precision and accuracy data for each QC sample. The repeatability and intermediate precision values (CV%) ranged from 1.9% to 7.4% and from 2.5% to 11.4%, respectively. The within-run and between-run accuracy values (recovery %) ranged from 95.4% to 109.5% and from 99.3% to 103.3%, respectively.

### Stability

The three freeze–thaw cycles (post-preparative, short-term, and long-term) did not cause any significant change in the vancomycin concentrations. The deviation values obtained for 10.0 and 100.0 µg/mL vancomycin solutions were 4.5% and 3.9%, respectively. For all stability tests of vancomycin in human plasma, the deviation values observed were <12%, as shown in Table II.

### Assay applicability

Seven samples from different patients were obtained to evaluate the applicability of the method. Clinical and laboratory data from patients are described in Table III.

Around 43% of the patients used at least 10 drugs simultaneously, with a mean of 8.85 per patient. The most reported drugs

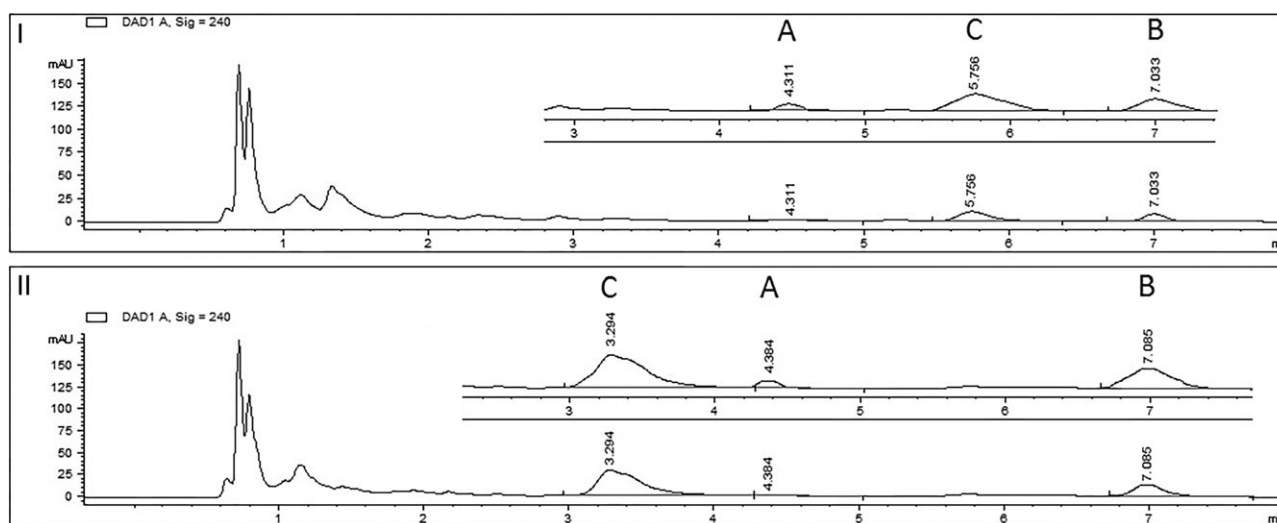
included antimicrobials, immunosuppressives and antihypertensives. Unknown exogenous substances were observed in the chromatograms of two patients, without interfering with the retention time of vancomycin and the IS, as shown in Figure 2.

### Discussion

The immunoassay techniques for vancomycin, such as FPIA, are simple and rapid and are widely applied for the therapeutic monitoring of this drug (15–18). Although the calibration curves are quite robust, problems related to these techniques are often reported as they may underestimate vancomycin levels in plasma (24–26). Moreover, for samples containing either low or high levels of vancomycin, FPIA has proved to be far less accurate than UHPLC techniques (27). Although other techniques have proven to be simpler and faster for vancomycin determination, HPLC has significant advantages because of its greater sensitivity and specificity when compared with immunoassays (20). The present method differs from other published HPLC methods as it is rapid, easy to perform, and requires minimal plasma aliquots, similar to the UHPLC method reported in the literature (27).

Protein precipitation with acetonitrile was used for sample preparation due to the lower cost when compared to solid phase extraction (SPE), as described in other studies (14, 28). SPE is relatively expensive as the cartridges are manufactured for single-use only. In addition, SPE requires much time for analysis (29), which is not effective for vancomycin therapeutic monitoring.

The chromatographic separation was performed on a C<sub>18</sub> column with a small particle size (2.7 µm) and shorter analysis time without loss of separation efficiency. Improved chromatographic resolution (30) is an advantage of our method. Most studies have reported the use of C<sub>18</sub> columns with 5.0 µm (14, 31, 32) or 10.0 µm (28) particle size. The mobile phase, with 20 mM acidified ammonium acetate buffer and methanol (88:12 v/v) in an isocratic elution system, makes it easier for the technique to be applied to several HPLC devices. In addition, isocratic HPLC is associated with relatively low cost for equipment and reagents when compared with those required for immunoassays (14). Although the gradient



**Figure 2.** Representative HPLC chromatograms of patient samples. Sample of the fifth patient (I): retention time of vancomycin (A): 4.3 min, IS (B): 7.0 min and unknown exogenous substance (C): 5.8 min. Sample of the seventh patient (II): retention time of vancomycin (A): 4.4 min, IS (B): 7.1 min and unknown exogenous substance (C): 3.3 min.

elution system has been reported in other studies (28, 31), its employment in hospitals is less feasible due to its cost, particularly in low-income countries.

Unlike Chauhan *et al.* and Usman *et al.* (31, 32), our group used an internal standard to improve the precision and accuracy of results. The elution of vancomycin and zidovudine (IS) is 4.0 and 7.1 min, respectively, and the running time of 8 min for each sample allows for a larger number of samples to be analyzed. The present method is faster when compared with those reported in previously studies (14, 28, 31), including a recent method in which vancomycin was eluted in 9.1 min (32).

Small plasma concentrations (50.0  $\mu$ L) for vancomycin quantification is another advantage of the present method, as opposed to other methods that require plasma amounts greater than 100.0  $\mu$ L (14, 27, 28, 32–34). This is relevant as most patients on vancomycin treatment have limited venous access. In addition, lower amounts of plasma lead to a reduction in the sample preparation time and allow for minimal use of solvents.

The sensitivity of the present method is similar (LoQ = 1  $\mu$ g/mL) to that of the methods reported by Hagihara *et al.* and Favetta *et al.* (14, 34) but less sensitive when compared to those of other studies (LoQ = 0.25  $\mu$ g/mL (28, 32) and 0.50  $\mu$ g/mL (19, 33)). For vancomycin therapeutic monitoring, the LoQ = 1  $\mu$ g/mL is sufficient once its use in clinical practice aims at achieving therapeutic concentration values in the range of 10–20  $\mu$ g/mL for the treatment of severe infections and for avoiding the development of bacterial resistance (10).

All the results for precision and accuracy were within the limits accepted by the Brazilian Health Surveillance Agency legislation and ICH guideline: CV% < 11.5 and recovery% ranged from 95.4 to 109.5. In our study, vancomycin was maintained stable at room temperature for 24 h after three freeze and thaw cycles and after freezing for more than 3 months at  $-20^{\circ}\text{C}$ , similar to that in a recent study (32).

Our method has some limitations. First, only a few samples from patients were used to evaluate the assay applicability. In addition, the limit of detection and the signal-noise ratio were not calculated.

## Conclusion

We have developed and validated a specific, sensitive, precise and accurate HPLC-DAD method for the quantification of vancomycin in human plasma within the expected concentration range. Our method is more rapid and simpler when compared to other HPLC methods. The small volume of sample required for analysis also makes the method adequate for the therapeutic monitoring of vancomycin, and therefore, a good alternative to existing methods.

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