

A new and ecological miniaturized method by spectrophotometry for quantification of vancomycin in dosage form

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Vancomycin, an important antibiotic, is marketed as lyophilized powder. In the context of routine analysis of this product, the existence of a more advantageous and effective method is interesting. Thus, the objective of this work is to develop and validate a new analytical method, faster, low-cost, ecological and miniaturized for quantification of vancomycin in lyophilized powder using spectrophotometry in ultraviolet region. Buffer solution pH 6.8, quartz cuvette with capacity of 700 μ L and 280 nm were chosen. The method proved to be linear in the range of 50-150 μ g/mL (0.9997). The selectivity of the method was proven in two ways: The standard-sample overlay aimed to identify vancomycin in the sample; The forced degradation test (sample solutions prepared in 0.01 M HCl, 0.01 M NaOH and aqueous conditions and kept at 60 °C by 8 hours, and UV 254 nm at ambient temperature during 24 hours) aimed to show the susceptibility of the method to consequently indicate the stability of the sample. It was precise in intraday (RSD 1.27 %), interday (RSD 1.18 %) and between analysts (RSD 1.92 %) levels. It was robust when small variations were performed in seven important parameters (wavelength, cuvette, filtration step, dibasic and monobasic phosphate brand, ultrasound time and source of water). The accuracy was proved by the standard recovery test and showed mean recovery of 101.10 %. This method can be applied in routine analysis of quality control of vancomycin lyophilized powder and it is an effective, accessible and ecological alternative, which follows the Green Analytical Chemistry principles, presenting less waste generation, no use of toxic solvents, smaller sample volumes and required diluents, which impacts on the final cost of the analyzes.

Keywords: ecological alternative, green analytical chemistry, lyophilized powder, spectrophotometry in ultraviolet region, vancomycin.

<https://doi.org/10.22456/2527-2616.112226>

Introduction

Vancomycin (Figure 1) is a glycopeptide antibiotic obtained naturally and the first uncovered in this class. Its mechanism of action comprises the disrupt of bacteria cell wall by interfering in the synthesis of the cell wall (1-2). It is indicated for the treatment of infections caused by Gram-positive bacteria, including Methicillin resistant *Staphylococcus aureus* (MRSA), and intestinal infections caused by *Clostridium difficile* (2-3). Due its low absorption through gastrointestinal tract, it is intravenously administrated to treat systemic infections (4). Only in case of diarrhea caused by *Clostridium difficile*, the oral via is required (5). It is mainly eliminated through renal tract, what can lead to nephrotoxicity in people with renal disease, making necessary to adjust the dosage (3). Due the development of MRSA, vancomycin became the only drug to be used in the treatment of infections caused by this microorganism (6). It is also used as an alternative in case of allergy to penicillin (7-8).

The development of analytical methods is of great importance for the quality control of drugs in pharmaceutical industries, it is a way to assure the safety and efficacy of the drug. The spectrophotometric method by UV is simple and easy to perform, besides it is low cost and provides precise results in quantitative analysis

of drugs (9-10). It was found some methods in literature for quantification of this drug using the spectrophotometry in UV region (11-17), however they do not bring the Green Analytical Chemistry proposal.

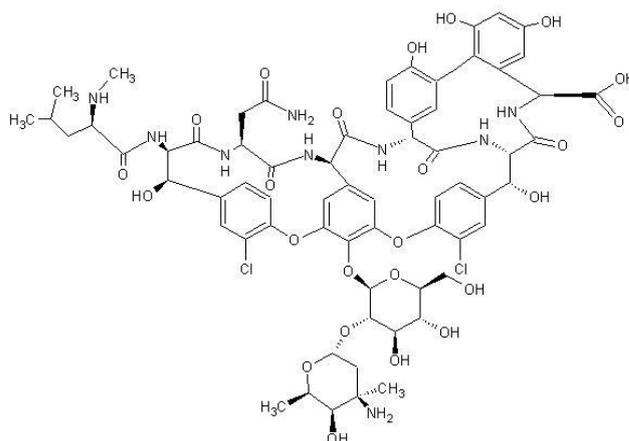


Figure 1. Chemical structure of vancomycin (CAS 1404-90-6).

According to the official compendia, British Pharmacopoeia (18) and American Pharmacopoeia (19) recommend HPLC and agar diffusion method. Japanese Pharmacopoeia (17) recommends UV (but not on a miniaturized scale and greater amount of drug 100 μ g/mL) and agar diffusion method. Besides the use of no

toxic solvents, it is possible to make these methods become greener, following the Green Analytical Chemistry principles (20-22). One alternative is the miniaturized analyzes, which are made using smaller amounts of sample and standard volume, as well as diluents, which generates less waste, makes the methods more economical and dynamic (23-27).

So, the objective of this work is to develop a miniaturized method by spectrophotometric in UV region for quantification of vancomycin in lyophilized powder.

Experimental

Materials and reagents

The reference standard (secondary standard) was vancomycin, content 96.3 %, and pharmaceutical form (sample) was vancomycin in lyophilized powder for injection 500 mg (labeled content). They were kindly donated by ABL Antibióticos do Brasil (Cosmópolis, São Paulo, Brazil).

The reagents used were purified water (water purification system (Millipore™), potassium phosphate dibasic (Sigma™) and monobasic (Synth™), to prepare the potassium phosphate buffer solution pH 6.8 used as diluent.

Equipment

The equipment used was a Spectrophotometer UV mini-1240 (Shimadzu™), quartz cuvettes with 1 cm optical path and capacity of 700 µL, analytical balance model DV215CD (Ohaus™), water purification system (Millipore™), and ultrasound equipment Ultrasonic Cleaner (Unique™).

Solution preparation

The solutions of vancomycin reference and sample were prepared in buffer pH 6.8. An amount of 5.19 mg of vancomycin reference was weighted and transferred to a 25 mL volumetric flask with buffer pH 6.8 and submitted to an ultrasound bath during five minutes to complete dissolution, and then completed the volume with the same diluent in order to obtain a reference solution of 200 µg/mL. This stock solution was filtered through a filter paper and an aliquot of 2.5 mL was taken and transferred to a 5 mL volumetric flask and completed with buffer, giving a solution of 100 µg/mL.

The sample solution was prepared considering the average weight from twenty vials of vancomycin (504.82 mg) and an amount of 5.05 mg of vancomycin sample was weighted and transferred to a 25 mL volumetric flask with buffer pH 6.8 and submitted to ultrasound bath during five minutes until complete dissolution, and then the volume was completed with the same diluent to reach a solution with concentration of 200 µg/mL. This stock solution was also filtered by a filter paper and an aliquot of 2.5 mL was taken and transferred to a 5 mL volumetric flask and completed with buffer, giving a solution of 100

µg/mL. Spectra were obtained from scanning in the wavelength range of 200-400 nm.

Ringbom curve

The Ringbom curve was obtained by the determination of the absorbance of 33 standard concentrations of vancomycin at 280 nm, in order to determine the linear concentration range.

An amount of 103.84 mg of vancomycin reference was weighted and transferred to a 200 mL volumetric flask with buffer pH 6.8 and submitted to ultrasound bath by five minutes, and then completed the volume with the same diluent to reach a solution of 500 µg/mL. This stock solution was filtered using a filter paper and the aliquots were taken in order to prepare 33 solutions, which varied through 10 to 500 µg/mL.

Validation parameters

The validation procedure was performed according to the International Conference of Harmonization (28) specifications for linearity, selectivity, limits of detection and quantification, precision, robustness and accuracy.

Method validation

Linearity

From the Ringbom curve 6 concentrations were chosen for evaluation of the linearity of the method. For this, 10.38 mg of vancomycin reference (equivalent to 10 mg of vancomycin) was weighted and transferred to a 50 mL volumetric flask with buffer pH 6.8 and submitted to ultrasound bath by five minutes, and then the volume was completed with the same diluent to reach a solution of 200 µg/mL. From this stock solution, aliquots were taken to prepare the solutions of 50, 70, 90, 110, 130 and 150 µg/mL. The absorbance was measured at 280 nm. The linearity was performed in three different days and in triplicate.

The data obtained were evaluated by regression analysis. The equation of the line was determined by linear regression analysis by the method of the least squares. The Analysis of Variance (ANOVA) was also performed for the absorbance values obtained in each concentration.

Selectivity

The selectivity of the method was determined by the response obtained for vancomycin solutions reference and sample. It was also proved by the forced degradation test, where the vancomycin solution was prepared in 0.01 M HCl, 0.1 M NaOH, H₂O₂ 0.3 % and in water at a concentration of 100 µg/mL and submitted to water bath at 60 °C. Aliquots of these solutions were taken at 2, 4, 6 and 8 hours and the absorbance were measured, as well as the absorbance profile. Additionally, a solution of vancomycin was prepared in buffer at a concentration of 100 µg/mL and submitted to UV light (254 nm), and

aliquots of this solutions were taken at 2, 4, 6, 8 and 24 hours and immediately analyzed.

Limits of detection and quantification

The limits of detection and quantification were obtained from the three calibration curves of the linearity, using the Equations 1 and 2, respectively:

$$\text{LOD} = 3 \times \frac{\text{SD}}{a} \quad \text{Equation 1}$$

$$\text{LOQ} = 10 \times \frac{\text{SD}}{a} \quad \text{Equation 2}$$

SD: standard deviation

a: average slope (obtained from the calibration curve from linearity)

Precision

Precision was evaluated by the repeatability and the intermediate precision. The repeatability was performed using six replicates of the same concentration (90 µg/mL) in the same day, with the same analyst, under the same conditions of analysis. The intermediate precision was performed by another analyst on a different day, under the same conditions of the method. The precision was analyzed by RSD (%) values. It was considered precise when RSD (%) values were lower than 2.0 %.

Robustness

The robustness of the method was performed according to Youden & Steiner test (29), where seven fundamental analytical parameters were chosen and small variations were performed. The parameters chosen were: wavelength, cuvette, filtration step, dibasic and monobasic phosphate brand, ultrasound time and source of water. The method was considered robust when the effects of each parameters was smaller than $\text{SD} \times \sqrt{2}$.

Accuracy

The accuracy of the method was demonstrated by the recovery test, in triplicate and in three levels, 80, 100 and 120%, considering 100 µg/mL (100%). A standard stock solution was prepared in the concentration of 1000 µg/mL. A sample solution was also prepared in the same concentration. From the sample solution, aliquots of 500 µL were taken and transferred to 10 mL volumetric flask (50 µg/mL). Then, from the reference stock solution aliquots of 300, 500 and 700 µL were transferred to 10 mL volumetric flasks containing the sample aliquot, in order to obtain a final concentration of 80, 100 and 120 µg/mL, respectively. The mean recoveries, expressed in terms of percentage of standard recovered and RSD (%), were determined. It was considered accurate when recovery was between 98-102% and RSD (%) values were smaller than 2.0% (30).

Content analysis

The solutions preparations were made in accordance with item 2.3. and absorbance was measured. The values of average absorbance for each solution were compared using the Equation 3.

$$C_s = A_s \times \frac{C_r}{A_r} \quad \text{Equation 3}$$

C_s: concentration of sample solution; *A_s*: absorbance of sample solution; *C_r*: concentration of reference solution; *A_r*: absorbance of reference solution.

It was considered adequate when content was between 90-115% (17).

Results and Discussion

Ringbom curve

Vancomycin spectrum in the region of 200 to 400 nm showed better absorption at a wavelength of 280 nm and it was used in the Ringbom curve to define the validation concentrations. In the comparison between purified water and buffer pH 6.8, as diluent, the buffer showed better absorbance values when the same vancomycin concentration was used.

The Ringbom curve obtained is shown in Figure 2. Six concentrations, 50 to 150 µg/mL, were chosen from this curve to perform the linearity of the method.

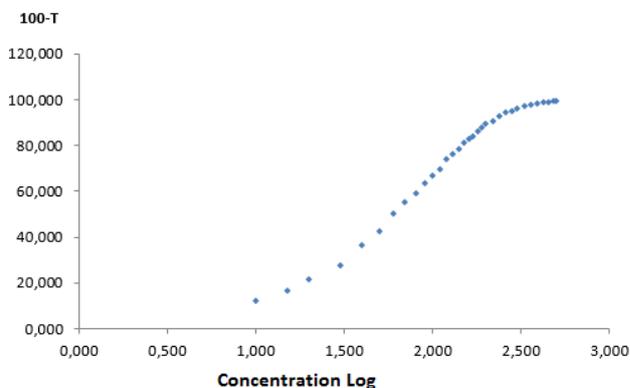


Figure 2. Ringbom curve of the proposed method.

Linearity

An appropriate amount of standard vancomycin was diluted in buffer pH 6.8 in order to obtain work solutions of 50-150 µg/mL. The data were validated by ANOVA (Table 1), which showed significant linear regression ($F_{\text{calculated}} > F_{\text{critical}}$, $p = 0.05$), and no significant lack of fit ($F_{\text{calculated}} < F_{\text{critical}}$, $p = 0.05$), in this way, the method can be considered linear.

Selectivity

The selectivity of the method was analyzed by comparing the spectra of vancomycin solutions reference and

sample, which overlapped perfectly and allowed to identify vancomycin in the analyzed sample (Figure 3).

Table 1. ANOVA results for linearity of the method proposed.

Parameters	280 nm
Linearity range ($\mu\text{g/mL}$)	50-150
Slope	0.0044
Intercept	0.0018
Correlation coefficient (r)	0.9994
Regression	1385.72 (4.75)
Lack of fit	0.44 (3.26)

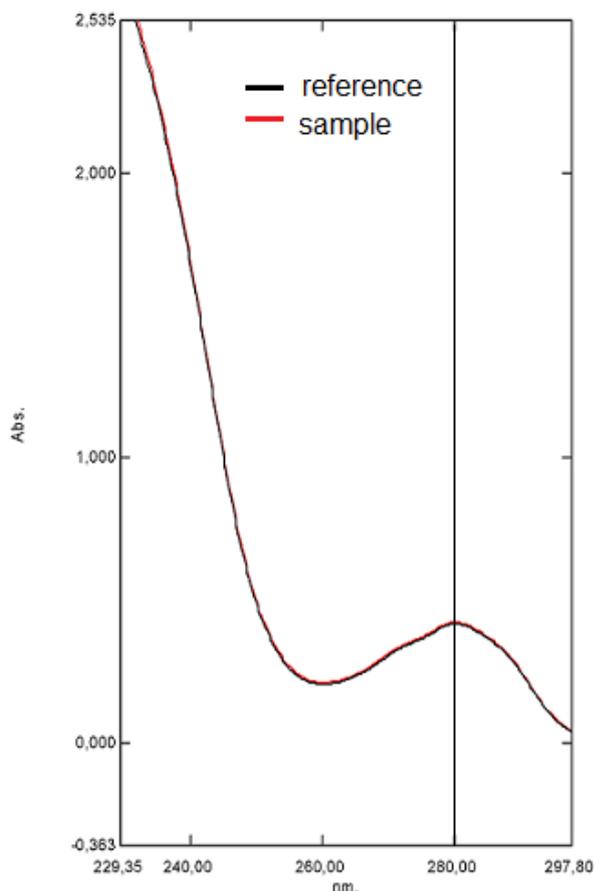


Figure 3. Overlap of vancomycin reference and sample spectra at a concentration of 100 $\mu\text{g/mL}$.

It was also analyzed by the spectra obtained in the forced degradation test. The vancomycin solution was submitted to acidic, alkaline, oxidative, aqueous and photolytic media and in all conditions the band of maximum absorbance of vancomycin was smaller when compared to the spectra obtained in time 0 (Table 2), showing the susceptibility of the method and its capacity to indicate stability. In a study by HPLC (31), vancomycin in lyophilized powder was degraded under the same forced conditions as this work and the results (after the same periods of time) also showed the susceptibility of the method and its ability to indicate the stability of the sample. After all, forced degradation studies challenge the

method, which must be able to reveal the true situation of the drug.

In this context, the spectrum obtained in the oxidative condition showed relevant tracing distortion, which may be due to the possible degradation of the drug and the appearance of degradation products, as revealed in the study by HPLC (31), or to the interference of the preservative present in the solution of H_2O_2 , also seen in other studies of forced degradation analyzed by UV (32-33). And, even in this context, the unchanged tracings of the other degradations may be suggestive of a small instability of the molecule (results shown in Table 2), which corroborates the results by HPLC (31).

Table 2. Absorbance values at 280 nm and percentual decrease of vancomycin sample after the degradation conditions employed, by the proposed method.

Degradation conditions	Abs time 0	Abs time 8 h/24 h*	Degradation percentage (%)
HCl 0.01 M – 60 °C	0.448	0.441	1.56
NaOH 0.1 M – 60 °C	0.450	0.422	6.22
H_2O_2 0.3% - 60 °C	0.626	0.595	4.95
H_2O – 60 °C	0.446	0.418	6.28
UV 254 nm	0.443	0.414	6.55

*24-hour time for photolytic degradation only.

It is valid to attest that the evaluation was made at 280 nm, which was the wavelength chosen in the development of the method for vancomycin. The spectra of each degradation in time 0 and after 8 hours, for acidic, alkaline, oxidative and aqueous degradation, and after 24 hours for photolytic degradation test are shown in Figure 4.

Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ obtained were, respectively, 0.72 and 2.19 $\mu\text{g/mL}$. The limits are low what shows the sensibility of the method.

Precision

Precision was proved by the repeatability showing RSD of 1.27 % and by intermediate precision showing RSD of 1.92 %. Both precisions showed adequate results of RSD (%) being smaller than 2.00 %, so the method can be considered precise. The results for repeatability and intermediate precision are shown in Table 3.

Robustness

The robustness of the method was performed by Youden & Steiner test (29). The effects, as well as the $\text{SD} \times \sqrt{2}$, are shown in Table 4. As the effects show a value below 3.25, the modifications studied in each parameter do not significantly change the method.

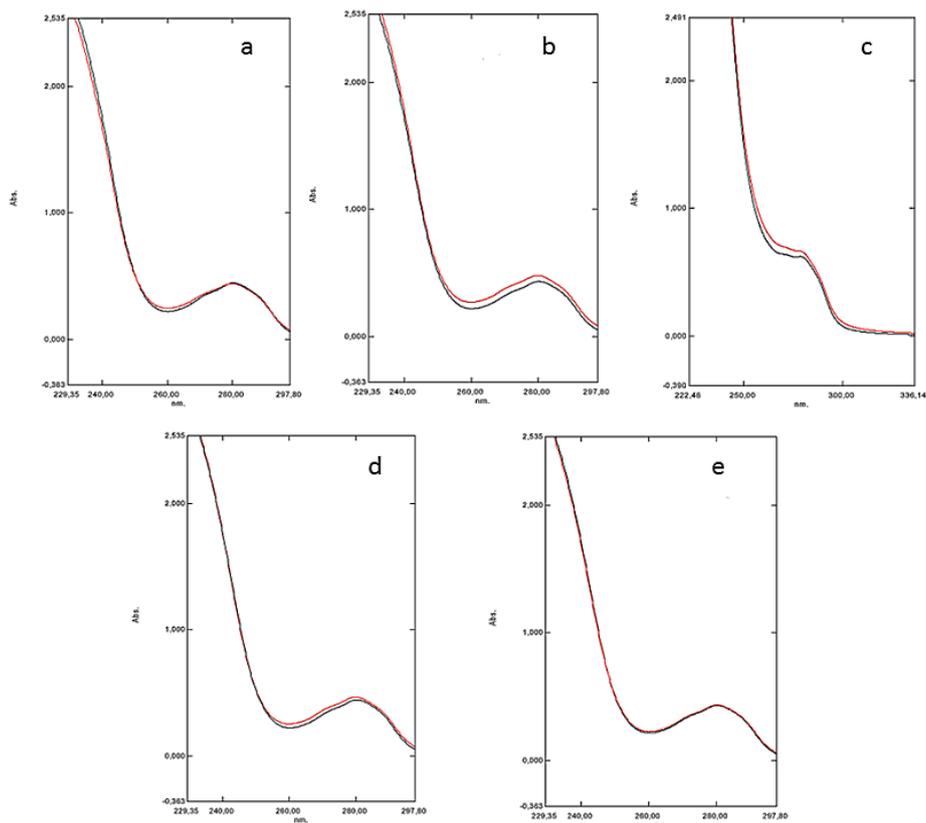


Figure 4. Spectra of vancomycin in time 0 (red) and time 8 hours (black) at acidic (a), alkaline (b), oxidative (c) and aqueous (d) conditions, and after 24 hours at photolytic (e) media, all at a concentration of 100 µg/mL.

Table 3. Precision results for miniaturized method by UV for vancomycin.

Wavelength	Level	Absorbance						RDS (%)
		1	2	3	4	5	6	
280 nm	Repeatability	0.400 ^a	0.410 ^a	0.407 ^a	0.409 ^a	0.416 ^a	0.407 ^a	1.27
	Intermediate	0.404 ^b	0.402 ^b	0.399 ^b	0.405 ^b	0.390 ^b	0.392 ^b	1.92
		0.390 ^c	0.400 ^c	0.390 ^c	0.392 ^c	0.395 ^c	0.389 ^c	

^a: Absorbance obtained by analyst 1 in one day of analysis; ^b: Absorbance obtained by analyst 1 in two days of analysis; ^c: Absorbance obtained by analyst 2 in two days of analysis.

Table 4. Robustness of the miniaturized method by UV for vancomycin.

Parameter	Normal	Changed	Effect	Global standard deviation	RSD x √2
Wavelength (nm)	280	282	2.84	2.30	3.25
Cuvette (mL)	0.7	2.5	-0.23		
Filtration	Yes	No	0.91		
Dibasic phosphate brand	Sigma	Qhemis	-1.70		
Monobasic phosphate brand	Synth	Vetec	1.70		
Ultrasound time (min.)	5	3	0.91		
Source of water	Lab 1	Lab 2	-1.70		

Accuracy

The accuracy of the developed method was determined by recovery of standard. The recoveries are shown in the Table 5, as well as the RSD (%) values. The method can be considered accurate, considering the mean recovery in three days of analysis, in accordance to 98-102%, as specified by AOAC and Horwitz trumpet (30, 34) and RSD (%) values below 2.00%.

Table 5. Recovery results for vancomycin using the developed spectrophotometric method.

	Vancomycin Standard added (µg/mL)	Vancomycin Standard recovered (µg/mL)	Recovery (%)	Mean recovery (%)	RSD (%)
R1	30	30.14	100.47	101.10	0.50
R2	50	50.79	101.57		
R3	70	70.87	101.25		

Content analysis

The content analysis of vancomycin sample is shown in Table 6. Dosing was carried out in three days and in triplicate in order to obtain the vancomycin content in the pharmaceutical product. In addition, it is possible to confirm that the content is in accordance with the established in the monograph, which establishes content of 90–115% (17). The average content of vancomycin obtained in the final product was 95.65% and is in line with the recommended.

Table 6. Content analysis for vancomycin lyophilized powder using the UV spectrophotometric method proposed.

Day	Average Content* (%)	Final Content (%)	RSD (%)
1	94.56		
2	96.12	95.65**	0.99
3	96.26		

* Average of 3 determinations.

** Content within the specification as recommended by the Japanese Pharmacopoeia of 90 to 115 % (17).

The UV spectrophotometric method developed for quantification of vancomycin was validated according to the parameters established in ICH guidelines (28). The method can be considered ecological, since:

- it uses no toxic solvents = buffer solution pH 6.8
- it uses less quantity of diluents, reagents and sample = 700 μ L
- it is a simple and easy method to be performed = spectrophotometry in the ultraviolet region
- it is a fast method, showing results in a short period of time = seconds

The analytical conditions chosen make the method advantageous and part of the Green Analytical Chemistry, being dynamic, miniaturized and ecological with a small generation of residues, which contributes positively to the health of the environment.

Conclusion

There is a gap in the literature of articles for the evaluation of vancomycin pharmaceutical products by UV on a miniaturized scale and that can be used in routine analyzes, as proposed. In the present work an effective, accessible and ecological alternative method by spectrophotometry in UV region was developed for vancomycin in lyophilized powder. It presents less waste generation, does not use toxic solvents, requires smaller sample volumes and diluents, which impacts on the final cost of the analyzes. The method proved to be linear in the range of 50 to 150 μ g/mL, selective, precise, robust and accurate, being a greener alternative that can be applied in the routine quality control process of vancomycin in pharmaceutical industries around the world.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgements

The authors acknowledge FAPESP for the financial support (Process 2018/02540-6) and ABL Antibióticos do Brasil.

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