Application of an Experimental Design for the Optimization and Validation of a New HPLC Method for the Determination of Vancomycin in an Extemporaneous Ophthalmic Solution

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Abstract

An experimental design has been used to develop and optimize a new high-performance liquid chromatographic (HPLC) method for the determination of Vancomycin in an extemporaneous ophthalmic solution. After the preliminary studies and literature review, the optimized method was carried out on a second generation of a C18 reverse-phase column (Luna 150 × 4.6 mm i.d., 5 µm particle size) and using methanol as organic phase, a less toxic solvent than acetonitrile, described in the extended literature. The experimental design consisted of a Placket-Burman design where six different variables were studied (flow rate, mL/min; temperature, °C; pH mobile phase; % buffer solution; wavelength; and injection volume) to obtain the best suitability parameters (Capacity factor-K', tailing factor, resolution, and theoretical plates). After the optimization of the chromatographic conditions and statistical treatment of the obtained results, the final method uses a mixture of a buffer solution of water–phosphoric acid (85%) (99.83:0.17, v/v) adjusted to pH 3.0 using triethylamine and mixed with methanol (87:13, v/v). The separation is achieved using a flow rate of 1.0 mL/min at 35°C. The UV detector was operated at 280 nm. The validation study carried out, demonstrates the viability of the method, obtaining a good selectivity, linearity, precision, accuracy, and sensitivity.

Introduction

Vancomycin (Figure 1) is a glycopeptide antibiotic initially obtained from *Streptomyces orientalis* cultures. It has a strong bactericidal activity, inhibiting the cell wall synthesis. Vancomycin is used principally for the treatment of severe infections caused by gram-positive bacteria in patients who cannot receive or who have failed to respond to penicillins and cephalosporins or for the treatment of gram-positive bacterial infections that are resistant to β-lactams and other anti-infectives (1). Parenteral dosage forms of vancomycin hydrochloride are used in the treatment of potentially life-threatening infections caused by susceptible organisms that cannot be treated with other effective, less toxic anti-infective agents.

Vancomycin is also usually used as an ophthalmic solution for the treatment of streptococcal endophthalmitis and corneal ulcers produced by gram-positive bacteria resistant to other antibiotics.

There are few commercial preparations of ophthalmic solutions due to its poor stability. It has to be prepared extemporaneously in the pharmacy hospital diluting vancomycin hydrochloride with artificial tears. Not many studies about the stability of these pharmaceutical forms have been performed (2–5), some of them using microbiological methods (6). A few papers (7–13) are published about the stability of vancomycin in different kinds of solutions and conditions.

There are several methods described based on high-perfor-

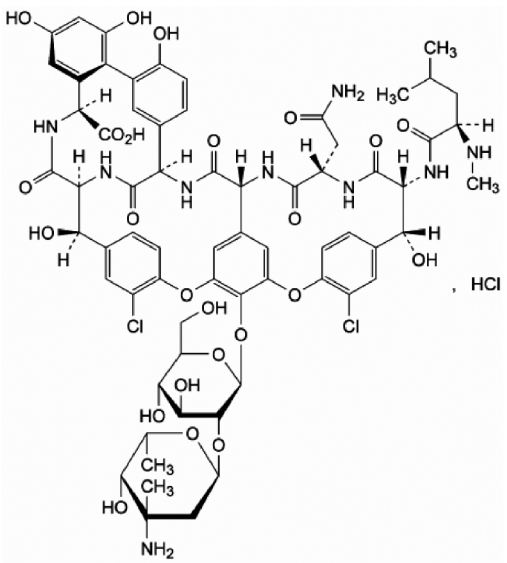

Figure 1. Chemical structure of vancomycin.

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mance liquid chromatography (HPLC) (14–27) for the determination of vancomycin in biological fluids because of the need of monitoring its levels due to its renal and auditory toxicity, even though most of these methods are time consuming (they require a complex method of extraction of vancomycin and long run times) owing to the complexity of a biological matrix. In addition, they use old, large columns and most of them use acetonitrile as organic solvent, which is a toxic reagent.

In the present work, a new method has been searched using a second-generation column (shorter, with little metal content and end-capped) and using methanol as organic reagent, as it is far more environmentally friendly than acetonitrile.

Afterwards, an experimental design is carried out in order to optimize the method found in the previous development. Based on the literature found (28–33), a Placket-Burman design is chosen as the selected method to realize this study.

Once the method has been established, a validation study is realized to demonstrate that the final method is suitable for our purpose (34–37).

The objective of this article is to develop, optimize, and validate a new HPLC method in order to study afterwards the stability of a specific ophthalmic solution of vancomycin.

Experimental

Equipment

An HPLC system consisted of a Hewlett Packard 1100 featuring a column oven (79856A), a quaternary pump (G1311A), an automatic injector (G1313A), and a diode array detector (DAD) detector (G1315A), which was set at 230 nm. Data acquisition was performed using a chromatography software package (Chemstation version A.07).

Materials and reagents

Methanol was of HPLC grade and was purchased from Panreac-Quimica SA (Barcelona, Spain). Phosphoric acid (85%) was purchased from Panreac-Quimica SA (Barcelona, Spain). Triethylamine was purchased from J.T. Baker (Barcelona, Spain). The water used was deionized and purified by means of a purelab Plus system by Vivendi.

Vancomycin HCl was purchased from Sigma-Aldrich (Barcelona, Spain).

Chromatographic conditions

Different trials based on the different literature described (38–43) were performed using several columns (with different hydrophobicity, particle size, metal content, and end-capped) and buffer solutions until the achievement of a preliminary method (Table I). It is necessary to take into account that vancomycin is a molecule with a high molecular weight, containing several polar groups (acidic, basic and neutral) as can be seen in Figure 1; therefore, the silanol activity is a parameter to consider seriously.

All buffer solutions are adjusted to pH 3.0 because phosphate buffer from pH ≥ 5 hastens the decomposition of vancomycin and it is demonstrated that the pH of maximum stability for vancomycin is between 3.0 and 5.0 (10). Combinations of the different buffers with methanol and the different columns were realized, and it was stated that the best column is Luna C18 and the most adequate buffer solution was water–phosphoric acid (85%) (99.83:0.17, v/v) adjusted to pH 3.0 with triethylamine. Different studies established that triethylamine improve the number of theoretical plates and symmetry factor (reduce the silanol activity and metal impurity effects) (41,43).

Finally, the nominal chromatographic conditions which will be optimized later on are described as the following: the separation was performed using a Luna C18 (2) column 150 × 46 mm i.d., 5 µm, made of stainless steel (Phenomenex, Torrance, CA). The mobile phase consisted of water–phosphoric acid (85%) (99.83:0.17, v/v) adjusted to pH 3.0 using triethylamine and mixed with methanol (85:15, v/v). Both methanol and buffer solution were degassed by filtering through a 0.45-µm GH-membrane filter. The flow rate was 1.0 mL/min. The DAD detector was operated at 280 nm. The injection volume was at 50 µL. The HPLC analysis was conducted at 40ºC. Each analysis required 20 min.

Working standard solution and resolution solution

Working standard solution of vancomycin was prepared at a concentration of 1.0 mg/mL by dissolving the appropriated amount of the compound in deionized water. This standard solution was used for the method validation.

The solution used to carry out the experimental design was the resolution solution described in the European Pharmacopoeia (44). A solution of Vancomycin HCl at 1.0 mg/mL (instead the described 0.5 mg/mL in order to achieve more sensitivity) was heated at 65°C for 24 h, then it was allowed to cool. The suitability parameters studied in the method optimization were calculated over this solution.

Test sample solution

The test solution used in the method validation was prepared at 1.0 mg/mL of Vancomycin HCl adding the necessary quantity of placebo to achieve the same ratio as it is present in the ophthalmic solution. This placebo contains deionized water and artificial tears (LIQUIFILM LAGRIMAS), an ophthalmic sterile solution, which consist of polyvinyl alcohol (14 mg/mL), benza-

Table I. Trials Realized Before Obtaining the Nominal Chromatographic Conditions to Optimize

Columns	Buffer Solutions
Hypersil ODS (3 µm, 10 × 0.46 cm)	KH_2PO_4 5mM, pH 3.0 with H_3PO_4 85%
Chromolith Performance RP-18e (10 × 0.46 cm)	H_2O–H_3PO_4 85% (99.83:0.17 v/v) pH 3.0 with triethylamine
YMC-Pack Pro C18, (5 µm, 15 × 0.46 cm)	H_2O–triethylamine (99.8:0.2 v/v) pH 3.0 with H_3PO_4 85%
Symmetry C18 (5 µm, 25 × 0.46 cm)	H_2O pH 3.0 with triethylamine
Luna C18 (2), 5 µm (15 × 0.46 cm)	

lkonium chloride (0.05 mg/mL), and other excipients as buffering agents, disodium edetate, chloride acid or sodium hydroxide, and purified water.

Optimization and Validation of the Method

Optimization of chromatographic conditions

Experimental design

Starting from the preliminar method, an experimental design was executed to find the most appropriate chromatographic conditions in order to obtain the best suitability parameters.

A two-level Plackett-Burman design (30,32,36,45) (often applied in robustness studies) has been chosen to observe the effects of the different factors on the suitability parameters selected.

Selection of factors and levels

The quantitative factors studied were selected from the description of the analytical method (46) and they are the ones that can influence most on the chromatographic behavior: flow rate, buffer solution percentage, buffer solution pH, column temperature, wavelength, and injection volume.

Table II. Factors and Levels Studied

Factor	Units	Level (–1)	Level (+1)	Nominal
Flow	mL/min	0.9	1.1	1.0
% buffer solution	%	83	87	85
pH buffer solution	–	2.8	3.2	3.0
Column temperature	°C	35	45	40
Wavelength	nm	275	285	280
Injection volume	µL	40	60	50

The different levels for each factor were selected symmetrically around the nominal value of the correspoding factor in the original method. The factors and their levels studied are shown in Table II.

Selection of the suitability parameters

The suitability parameters selected were the ones that ensure the adequate performance of the chromatographic system (35, 44): capacity factor (k'), tailing factor (T), number of theoretical plates (*N*), and resolution (*r*).

Carrying out of runs

A total of 12 experiments were obtained with this experimental design (see Table III) where the different levels of the factors are combined statistically.

The solution prepared to perform the study was the resolution solution described previously. The parameters capacity factor, tailing factor, and theoretical plates were studied over the vancomycin peak, and the parameter resolution was calculated between the peak of vancomycin and the nearest peak of a degradation product.

Five replicates of this solution were prepared; each replicate was analyzed following each of the resulting experiments.

Statistical analysis

The aim of the study is to determine which factor or factors have a significant effect (95% probability) on the suitability parameters as well as checking if this effect will have positive or negative repercussion on the parameter. A Statgraphics v. 5.1 software is used to carry out this study.

Validation study

The validation study was carried out according to corresponding ICH guidelines (34).

Stability of standard and test solutions

The stability at room temperature of standard and test solution was studied in order to establish the fraction of time during

Table III. Experimental Design Carried Out

	Level	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6	Trial 7	Trial 8	Trial 9	Trial 10	Trial 11	Trial 12
Flow	+1		X		X		X			X	X	X	
	–1	X		X		X		X	X				X
% buffer	+1	X		X			X		X	X	X		
solution	–1		X		X	X		X				X	X
pH buffer	+1	X	X	X	X	X	X						
solution	–1							X	X	X	X	X	X
Column	+1	X	X			X		X		X	X		
temperature	–1			X	X		X		X			X	X
Wavelength	+1	X	X	X		X	X	X			X	X	
	–1				X				X	X			X
Injection	+1	X	X				X	X	X			X	
volume	–1			X	X	X				X	X		X

which these solutions don't loose activity significantly and degradation products do not appear.

Different vials of each solution were prepared and injected alternatively at times: 0, 1, 2, 3, 8, 10, 12, 18, and 24 h and the experimental concentration was calculated comparing the areas at each time with the initial areas (time zero).

Specificity

In order to measure the specificity of the method, it was studied in the absence of interference by the excipients, which take part in the pharmaceutical preparation (placebo solution), as well as the absence of interference of the degradation products from vancomycin.

The following solutions were prepared and injected according to the analysis method obtained in the optimization: deionized water solution, placebo solution in deionized water at the test solution concentration, standard solution, standard solution spiked of the suitable quantity of placebo, and standard solution spiked of placebo and degradation products (these degradation products are generated heating the solution at 65°C for 24 h).

The purity of vancomycin peak is studied, as well as the absence of interference by the solvent, excipients, and degradation products by means of visual method.

Table IV. Experimental Design Results for Each Response Studied

Trial	k'	T	R	*N*
1	3.604	1.122	3.043	12716
2	0.567	1.038	0.734	2558
3	6.100	1.423	4.690	15298
4	1.105	1.367	1.270	10571
5	0.954	1.634	0.611	7855
6	4.724	1.477	4.215	11819
7	0.984	1.629	0.673	6561
8	6.407	1.661	4.439	11210
9	2.924	1.089	3.551	14038
10	2.911	1.091	3.535	14044
11	1.015	1.341	0.917	4037
12	1.687	1.406	1.599	10741

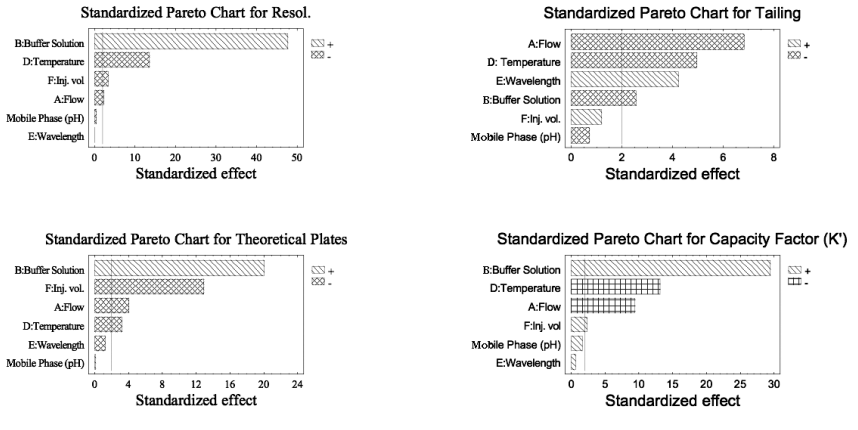

Figure 2. Pareto charts for the suitability parameters.

Linearity

Seven levels of standard solution spiked of the suitable quantity of placebo were prepared within the range of 70% and 130% of the working concentration (1 mg/mL).

The analysis was performed in triplicate, prepared individually, weighing the standard and placebo.

The experimental results were represented graphically, obtaining a calibration curve and carrying out the corresponding statistic study (ANOVA).

Precision

Instrumental system precision. The same standard solution was injected 10 times according to the analysis method to study the repeatability of the instrumental system. Furthermore, the intermediate precision of the system is studied, evaluating the variation of the responses between two different days.

Method precision and intermediate precision. Three levels of test solution were prepared within the range of 70% and 130% of the working concentration.

The solutions are prepared by weighing individually the standard and placebo and each level by triplicate. The relative standard deviation RSD obtained for the response factor was calculated.

Moreover, the intermediate precision was also checked preparing 7 replicates of test solution, previously mentioned, where the variability between analysts and days were studied.

Accuracy (recovery study)

The recovery method was studied at concentration levels of 70% (three samples), 100% (three samples), and 130% (three samples) where a known amount of the active was added to a determined amount of placebo, and it was calculated the quantity of vancomycin recovered in relation to the added amount.

Results and Discussion

Optimization of chromatographic conditions

The result of each suitability parameter was calculated for each experiment as they are shown in Table IV. The effect of each factor, if it has significative influence with a probability of 95%, on the suitability parameters, was calculated by the software described in statistical analysis section.

The different Pareto charts for each suitability parameter are shown in Figure 2. All the factors and their effect on the parameter can be seen in each chart. In these charts, a positive effect means that the factor produces an increase in the result of the parameter, and a negative effect means that the factor produces a decrease on the result.

Capacity factor

The factor with more significative influence was the buffer solution percentage, followed by column temperature, and flow rate, but

these two last with less importance. Injection volume has a minor influence, and pH and wavelength have no significative influence.

The optimum value of capacity factor had been established as 5. Based on the results of the software applied, the combination of the factors that achieve this optimum result established were 1.0 mL/min for flow, 87% buffer solution, 3.1 for pH value, 37°C of temperature, 277 nm, and 49 µL injection volume.

Tailing factor

The flow rate was the factor with the most significative influence, followed by column temperature, and wavelength. Buffer solution percentage had a little influence, and injection volume and buffer solution pH had no influence.

The best result for the tailing factor is 1, this means the peak is symmetric. It is found that the optimum combination of the factors that produce this theoretical result for the tailing factor were 1.1 mL/min for flow, 87% buffer solution, 3.0 for pH value, 45°C of temperature, 275 nm, and 40 µL injection volume.

Resolution

Buffer solution percentage was the factor with higher significative influence, as column temperature and injection volume but with less influence. It could be considered that flow, pH and wavelength had no influence on this parameter.

It is desirable to achieve the highest result for the resolution in order to guarantee a good separation between peaks. The best combination of the factors that provide a higher value of resolution were 0.9 mL/min for flow, 87% buffer solution, 2.8 for pH value, 35°C of temperature, 284 nm, and 40 µL injection volume.

Number of theoretical plates

The factors with more significative influence were buffer solution percentage and injection volume. Buffer solution percentage had influence either in retention time as in the width of the peak, both parameters with influence in the estimation of theoretical plates. Flow and column temperature had very low influence, and pH and wavelength had no influence.

This parameter is indicative of the column efficiency. In this way, the highest result for the theoretical plates must be achieved, suggesting a good chromatographic method. The optimum combination of the factors in order to obtain the highest number of theoretical plates were 0.9 mL/min for flow, 87% buffer solution, 3.1 for pH value, 35°C of temperature, 275 nm, and 40 µL injection volume.

Discussion

Taking into account the suitable results obtained for each of the studied responses, the optimum chromatographic method had been established to be validated later on.

The only factor that doesn't have any influence in the suitability parameters was the pH of the buffer solution. For this reason, the value was maintained as in the initial method, at 3.0. Wavelength had an insignificant influence on the tailing factor, but its value was set at 280 nm because this is the maximum absorption of vancomycin.

The optimum percentage of buffer solution was in all cases 87%, so this the value set in the definitive method.

In the case of the temperature, 35°C was the best value found for all parameters except for tailing factor. It was preferable as it was favorable to three of the parameters, even though for the tailing factor it had a negative influence. For this reason, 35°C was the final temperature for the method.

Regarding the factor injection volume, 40 µL was the suitable volume found for resolution, tailing factor, and number of theoretical plates; however, a higher volume was found to be better for capacity factor. It was also preferable as a good value in the other parameters rather than capacity factor, as this parameter only has influence in the time of the chromatogram. For these reasons, 40 µL was the set value chosen for the final method.

Finally, different values of flow rate were found as the best for each parameter. For tailing factor, the suitable value of flow was 1.1; nonetheless, a value of 0.9 was found the best value for resolution and theoretical plates. The final value chosen is 1.0, as this

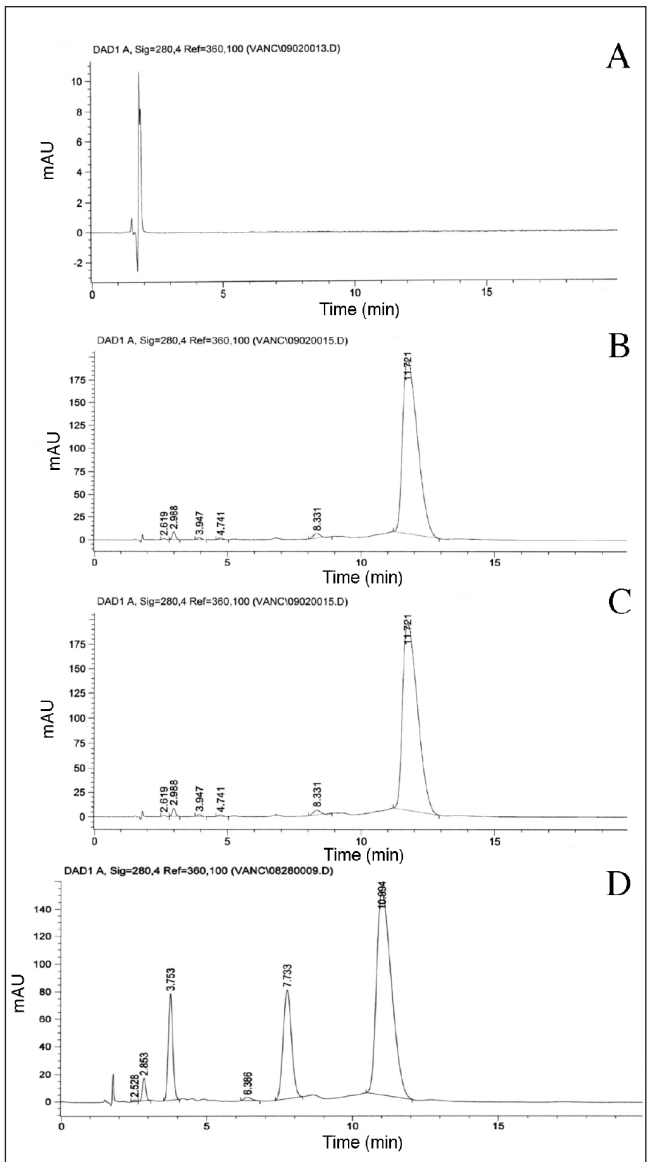

Figure 3. Representative chromatograms obtained from the specificity study. A placebo sample (A); a standard solution with vancomycin (B); a placebo sample spiked with vancomycin (C); chromatogram showing placebo, vancomycin, and its degradation products (D).

was an intermediate value and it could have a minimum effect on the parameters.

To conclude, the definitive conditions of the chromatographic separation was performed using a Luna C18 (2) column 150 × 46 mm i.d, 5 µm. The mobile phase consisted of a buffer solution of water–phosphoric acid (85%) (99.83:0.17, v/v) adjusted to pH 3.0 using triethylamine and mixed with methanol (87:13, v/v). The flow rate was 1.0 mL/min. The DAD detector was operated at 280 nm. The injection volume was 40 µL. The column temperature was 35°C. Each determination required 20 min (retention time of vancomycin was approximately 10 min).

Validation study

Stability of standard and test solutions

The study of the stability of standard and test solution indicates that both solutions are stable during 24 h because all the times studied in this period of time remain within the established limit of 95% of vancomycin, under which it is considered that the solutions were not stable. It can be concluded that the solutions can be stored at 25°C for a minimum period of 24 h.

Specificity

In Figure 3 it is observed that excipients as well as degradation products elute at different retention times and do not interfere between them.

Moreover, a purity test of the compound was performed, indicating that the three spectrums obtained at different times are within the threshold fixed for this study, 990.

To conclude, it can be stated that it was a selective method and suitable for routine work.

Linearity

Linear relationship is obtained between the peak area of vancomycin and the corresponding concentration in the concentration range of 0.7–1.3 mg/mL. The equation obtained was $y = 7782.8x - 309.2$ and the statistical parameters were 0.9991 for the correlation coefficient and 0.9982 for the determination coefficient. Also it was studied the variability (RSD) of the response factor (relationship between the response obtained and concentration) obtained a value of 0.97%. All results indicate a good linearity according with different specifications (45).

Precision

For the study of the instrumental system precision, the value of intra-day RSD of the areas obtained was 0.18% (day 1, $n = 10$) and 0.15% (day 2, $n = 10$). These values are far below 1%, the limit percentage set [Asociación Española de Farmacéuticos de la Industria (AEFI)] (45), thus showing that the equipment used for the study works correctly, being highly repetitive.

The maximum value of RSD obtained for the precision of the method was 1.43 % ($n = 7$), further below the maximum value of 2.7% established by Association of Official Analytical Chemists (AOAC) (47) and AEFI (45), hence, the method is repeatable.

The values of RSD for the inter-day and inter-analyst precision were 1.31% ($n = 14$) and 1.58% ($n = 14$) respectively, both of them below the established limit of 2.7% according to AOAC (47) for products with analyte concentration of 2.5% as it is the ophthalmic solution of this work, showing the method has a good intermediate precision.

Accuracy

The recovery obtained, individually and the mean of all the samples (100.48%) were between the 97.0–103.0% established according to the AOAC (47) for products with this analyst concentration. The average of the relative error calculated was 0.92.

Other statistic parameters were studied, as $t_{student}$ (1.2045 < 2.306 theoretical, stating that there was no significative differences between the average recovery and the value 100%) and $G_{Cochran}$, (0.5585 < 0.8709 theoretical, showing that the variances of the three concentrations were equivalents, that is, concentration does not have influence on the variability of the results). For all these reasons, it can be concluded that the method is sufficiently accurate.

Conclusion

A new simple and quick, HPLC method has been developed to be applied in routine to determine vancomycin in final product.

This method is optimized using a Plackett-Burman factorial design, obtaining the best method with suitable chromatographic parameters.

The proposed method has been evaluated over the linearity, precision, accuracy and specificity in the studied range (0.7–1.3 mg/mL) and proved to be convenient and effective for the quality control of vancomycin in the pharmaceutical dosage form studied (extemporaneous ophthalmic solution).

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