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PAPER

Development and validation of a new and rapid HPLC for determination of lyophilized teicoplanin†‡

Mariana Henrique Passoni and Hérida Regina Nunes Salgado*

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A sensitive, precise and specific high performance liquid chromatographic method was developed for the assay of teicoplanin in injectable pharmaceutical form. Analytical parameters were studied according to the International Conference on Harmonization (ICH). The method validation parameters yielded good results and included the range, linearity, precision, accuracy, specificity and recovery. The HPLC separation was carried out by reversed phase chromatography on a Waters symmetry C_{18} column (250 × 4.6 mm id, 5 μm particle size) with a phase composed of acetonitrile : methanol (50 : 50, v/v), pumped isocratically at a flow rate of 1.0 mL min^{-1}. The effluent was monitored at 279 nm. The developed HPLC method to determine lyophilized teicoplanin can be used to evaluate the quality of regular production samples.

1. Introduction

Teicoplanin (Fig. 1) is a new glycopeptide antibiotic produced by *Actinoplanes teichomyceticus*, which shows activity against staphylococci and streptococci. The complex consists of a mixture of five major components designated A2-1, A2-2, A2-3, A2-4, and A2-5 and one more polar component designated A3-1; minor components are also present. All teicoplanin components are glycopeptide analogs with molecular weights that range from 1564.3 to 1907.7.[1,2]

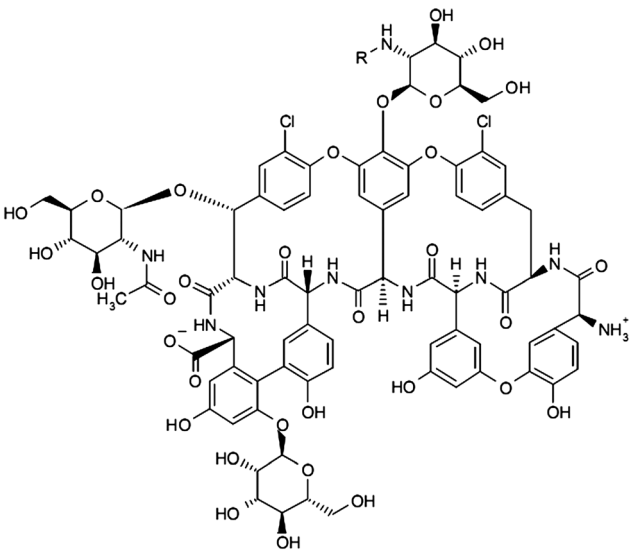

Fig. 1 Chemical structure of teicoplanin.

Teicoplanin can be administered extravascularly due to its good bioavailability, allowing its use in sequential therapy in patients requiring prolonged treatment. The glycopeptides have an adequate distribution in extra-cellular tissues, even teicoplanin, due to the balance between the fractions that are bound and unbound to plasma proteins and its long half-life. The elimination is almost exclusively renal.[3]

This antibiotic is one of the glycopeptides currently used in clinic for the treatment of multiresistant infections by Gram-positive organisms. It is commonly used in the treatment of endocarditis, dialysis-associated peritonitis and serious infections due to *Staphylococcus aureus*.[1,4,5]

Methicillin-resistant *S. aureus* is a global problem and has emerged as an important cause of hospital-acquired central nervous system infections. Although the main therapeutic choice is vancomycin, there are several reported cases treated with intrathecal or intravenous teicoplanin.[6]

Teicoplanin is comparable to vancomycin in terms of activity but presents pharmacokinetic advantages, such as prolonged half-life (88–182 h), allowing once daily administration and intramuscular injection, and a lower frequency of nephrotoxicity and ototoxicity; the teicoplanin has lower toxicity than vancomycin.[2,5]

Several analytical procedures are available in the literature for the analysis of teicoplanin in biological fluids by fluorescence polarization immunoassay, microbiological assays, and high performance liquid chromatography.[7–16] However, microbiological assays and immunoassays are labour intensive and time

Programa de Pós-graduação em Ciências Farmacêuticas—Faculdade de Ciências Farmacêuticas de Araraquara UNESP, Univ Estadual Paulista, Rodovia Araraquara-Jau, km 1—CEP, 14801-902 Araraquara, SP, Brazil. E-mail: salgadoh@fcfar.unesp.br

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consuming, and chromatographic methods for this drug showed a high retention time. Although teicoplanin has been studied in terms of therapeutic activity and commercialized, there is a non-official pharmacopoeial monograph for its quantification using HPLC and there are no studies describing HPLC quantitation methods of this drug in injectable forms.

HPLC is more widely employed because of its good specificity, sensitivity and cost effectiveness. This research aims to develop and validate a sensitive, precise, accurate and specific HPLC method for analysis of this drug.

2. Experimental

2.1. Chemicals and reagents

Teicoplanin reference substance and teicoplanin lyophilized injectable form were kindly supplied by Laboratório Cristália (Itapira, SP, Brazil). HPLC grade methanol was purchased from Merck (Darmstadt, Germany). HPLC water was prepared using Millipore Milli-Q® UFPlus apparatus (Millipore, Milford, MA, USA) and meets USA Pharmacopoeia requirements. All other chemicals were of analytical grade. The solutions were made according to USP Pharmacopoeia 2008. All solutions were filtered through a hydrophilic Millipore filtration membrane (13 mm, 0.45 μm pore size).

2.2. Equipments and chromatographic conditions

Quantitative HPLC was performed on a Waters Binary HPLC Model 2487 chromatograph equipped with a dual λ absorbance detector (set at 279 nm). The analytical column was a C18 Waters Symmetry (250 mm × 4.6 mm id, 5 μm particle size) column. The mobile phase used was methanol : acetonitrile (50 : 50, v/v). All analyses were done under isocratic conditions at a flow-rate of 1.0 mL min^{-1} and at room temperature. The mobile phase was degassed for 15 min at vacuum and filtered through 0.45 μm, 47 mm PTFE Waters Corporation (Milford, MA, USA) filtration membrane. The injection volume was 10 μL. The analysis required approximately 3 min. Peak identity was confirmed by retention time comparison. Data were analyzed by using the Microcal® Origin 6.0 software (Northampton, USA).

2.3. Preparation of stock and standard solutions

A quantity of teicoplanin reference substance equivalent to 50 mg was accurately weighed and transferred to 100 mL volumetric flask and water was added to make up the volume to give each one a final concentration of 500 μg mL^{-1}. The stock solution of teicoplanin was diluted with the mobile phase as needed to prepare different work standard solutions (70, 80, 90, 100, 110 and 120 μg mL^{-1}).

Ten injectable vials of teicoplanin were weighed and the average weight was calculated. A quantity of teicoplanin sample, equivalent to 50.0 mg of this drug, was transferred to 100 mL volumetric flasks. Then the water was added and shaken, and more water was added to give a final concentration of 500 μg mL^{-1}. This solution was filtered through a 0.45 μm membrane filter (Millipore) and was diluted with the mobile phase to prepare the working sample solutions. The solutions were prepared under identical conditions protecting from light.

2.4. Method validation

Method validation was performed following Association of Official Analytical Chemists[17] and ICH Guidelines (2003)[18] for system suitability, selectivity, linearity, precision, accuracy, detection limit, quantitation limit and robustness. The accuracy and precision of the assay as well as the linearity of the calibration curve were determined for intra- and inter-day on three different days. The precision was expressed as the percent coefficient of variation of each curve.

The iv solutions, containing selected excipients in the same proportion as in the final formulations, were used to validate the method for specificity and recovery.

2.4.1. Linearity. In order to assess the validity of the assay, 50 mg teicoplanin reference substance was dissolved in distilled water in 100 mL volumetric flask (500.0 μg mL^{-1}). Appropriate aliquots of this solution were diluted with the mobile phase, yielding concentrations of 70.0, 80.0, 90.0, 100.0, 110.0 and 120.0 μg mL^{-1}. Triplicate preparations of each concentration were performed.

2.4.2. Precision. Precision data for this validation were determined as recommended by ICH[18] (2003). Repeatability was calculated by assaying samples of 100.0, 110.0 and 120.0 μg mL^{-1} intra- and inter-day in three different days; results must be less than 5%. The RSD of peak areas ($n = 6$) was evaluated.

2.4.3. Accuracy. The accuracy of the method was evaluated by addition of three different amounts of teicoplanin reference standard solution (4.5, 9.0 and 13.5 μg mL^{-1}) to sample solution (90.0 μg mL^{-1}). At each level, samples were prepared in triplicate and the recovery percentage was determined.

2.4.4. Limit of detection and limit of quantitation. The limit of quantitation (LOQ) and limit of detection (LOD) were based on the standard deviation of the response and the slope of the constructed calibration curve ($n = 3$), as described in International Conference on Harmonization guidelines Q2 (R1). LOD and LOQ were determined according to eqn (1) and (2), respectively.

$$\mathrm{LOD} = \frac{3\sigma}{S} \quad (1)$$

$$\mathrm{LOQ} = \frac{10\sigma}{S} \quad (2)$$

where σ is the standard deviation of the responses and S is the calibration curve slope.

2.4.5. Selectivity. The selectivity of the method was performed by analyzing standard solutions of teicoplanin added of 20% extra of excipient, NaCl, reagent. Each solution was injected into the HPLC system in triplicate and the method is considered selective if an RSD ≤ 2.0% among peak areas is obtained. The chromatograms of the standard solutions with extra excipient were compared with the chromatograms obtained by standard solutions. The chromatograms were examined for possible interference.

2.4.6. Robustness. Robustness can be established by changing the chromatographic system as column, flow rate and mobile phase proportion. Each assay was performed in triplicate. Robustness of the developed method was indicated by the overall %RSD between data at each variable condition.

The robustness of the assay method was established by introducing small changes in the HPLC conditions which included wavelength (277 and 281 nm) and percentage of methanol in the mobile phase (52 and 48). Robustness of the method was studied using six replicates at a concentration level of 90.0 μg mL^{-1} of teicoplanin.

2.5. Calculations

Having established the quantitative relationships among parameters studied, and knowing the predictive performance of their association model, a linear simple regression by the least squares method was applied.

3. Results and discussion

Since, there are no methods describing quantitation of teicoplanin in pharmaceutical preparations, the goal of this study was to develop an HPLC assay for the analysis of this drug in lyophilized pharmaceutical form. Therefore, a method was developed and validated by linearity, accuracy, repeatability, specificity and robustness. For drug analysis in quality control, the simplest and fastest procedures can be applied. LC is a widely used method for analysis of antibiotics.[19–27]

Although teicoplanin has been studied in terms of therapeutic activity, there is only one official pharmacopoeial monograph in the Japanese Pharmacopoeia describing its quantification by microbiological assay.[28]

In this study, the chromatographic conditions were influenced by the physical–chemical properties of teicoplanin, such as solubility, polarity and UV absorption. The optimum mobile phase was obtained with acetonitrile–methanol (50 : 50, v/v). This mobile phase allowed the elution of teicoplanin with adequate retention time (1.8 minutes) (Fig. 2). The retention time (t_R) repeatability during the precision studies was found to be excellent for all the solutions. The symmetry of the peak was calculated as 1.39. The sharp and symmetrical peak was obtained with good baseline resolution and minimal tailing, thus facilitating accurate measurement of the peak area ratio. The chromatogram of the sample peaks was matching with the corresponding chromatogram of the standard drug peaks, which shows that the peaks of teicoplanin were pure and also formulation excipients were not interfering with the drug peaks. The wavelength of 279 nm was selected in order to permit the correct determination of teicoplanin after a UV spectrophotometric analysis.

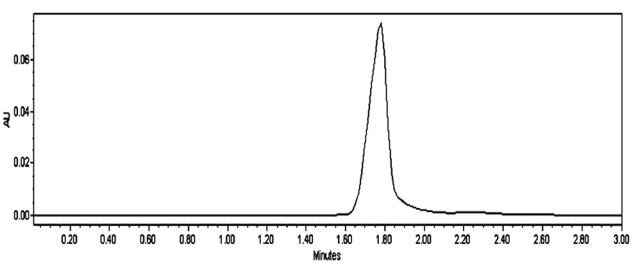

Fig. 2 Chromatogram of teicoplanin reference substance. *Conditions*: acetonitrile and methanol (50 : 50, v/v) as mobile phase, flow rate at 1.0 mL min^{-1}, at room temperature (20 ± 1 °C), C18 Waters Symmetry (250 mm × 4.6 mm id, 5 μm particle size) column at 279 nm and 10 μL of injection volume.

Table 1 HPLC conditions for determination of teicoplanin in powder for injection

System	Parameters
Apparatus	1525 Binary HPLC
Mobile phase	Methanol : acetonitrile (50 : 50)
Column	Waters C_{18} (WAT 0544275)
Wavelength	279 nm
Flow rate	1.0 mL min^{-1}
Volume of injection	10.0 μL
Temperature	20 ± 1 °C
Retention time	1.8 min

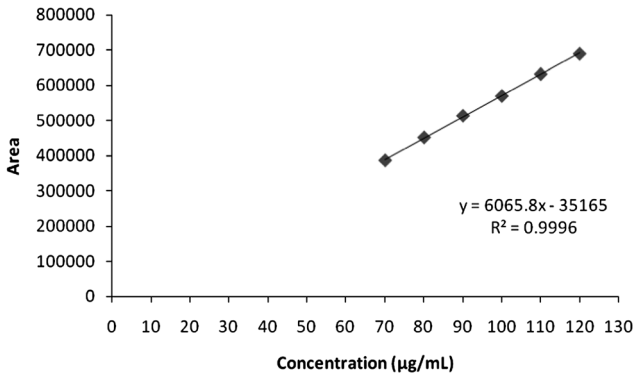

Fig. 3 Calibration curve constructed for teicoplanin standard solutions at 6 concentration levels in the range of 70 to 120 μg mL^{-1}.

Table 2 Intra and inter-day variations of the HPLC method for determination of teicoplanin

Concentration/μg mL^{-1}	Area ± s.d.[a]	RSD[b] (%)
100.0	557 574.9 ± 4439.68	2.39
110.0	62 018.1 ± 3735.93	1.81
120.0	678 110.4 ± 2944.05	1.30

[a] Mean of nine replicate analysis, s.d. = standard deviation. [b] RSD %—percent standard relative deviation.

Table 3 Experimental values obtained in the recovery test for teicoplanin samples, by the HPLC method

	Added/μg mL^{-1}	Recovered[a]/μg mL^{-1}	Recovery (%)
R_1	4.5	4.62	102.67
R_2	9.0	9.15	101.67
R_3	13.5	13.85	102.59

[a] Mean of three replicates analysis.

Table 4 Experimental values obtained for the determination of teicoplanin in powder for injection by the HPLC method[a]

Assays	Area	Found/mg	Assay (%)	Mean ± s.d.	RSD (%)
1	389 696 387 502 385 050	200.56	100.28	387 416 ± 1341.87	0.60
2	374 776 375 138 382 636	195.44	97.72	377 516 ± 2561.80	1.17
3	381 047 387 326 383 237	198.72	99.36	383 870 ± 1840.02	0.83

[a] s.d. = standard deviation, RSD%—percent standard relative deviation.

Validation of the method was performed according to the International Conference on Harmonization ICH (2003).[8] HPLC conditions for determination of teicoplanin in lyophilized are shown in Table 1. Linearity was performed by preparing calibration graphs. The calibration curves for teicoplanin were constructed by plotting concentration *versus* peak area and showed good linearity in a concentration range from 70.0 to 120.0 μg mL^{-1} of teicoplanin (Fig. 3). The regression equation was calculated by the least-squares method. The representative linear equation was $y = 6065x - 35\,165$, where x is the concentration of teicoplanin in μg mL^{-1}. The correlation coefficient was 0.999. Moreover, the relative standard deviations (RSDs) on the basis of the peak area ratios for three replicate preparations at each concentration level were found to be less than 2.0%.

The method was validated by evaluation of intra and inter-day precision. In the range of 100.0–120.0 μg mL^{-1}, the relative standard deviation (RSD) on the basis of the peak area ratios for three replicate preparations was found to be between 1.3 and 2.39% showing good results, as shown in Table 2. The recovery tests were performed according to the Association of Official Analytical Chemists AOAC (1990).[17] The mean absolute recovery determined by adding known amounts of teicoplanin reference substance (4.5, 9.0, 13.5 μg mL^{-1}) to the samples at the beginning of the process was found to be 102.31%. The experimental values obtained for the determination of accuracy are shown in Table 3. Recovery tests confirmed the accuracy of the proposed method and there was no excipients interference.

LOD and LOQ, calculated using the standard deviation of the responses and the slope of calibration curve, were 1.46 and 4.88 μg mL^{-1}.

Teicoplanin lyophilized forms were analyzed and the results obtained can be seen in Table 4. No interference from excipients could be observed at the detection wavelength (279 nm). Teicoplanin was shown to be stable during all the procedures. Besides Japanese Pharmacopoeia[28] recommends bioassay for teicoplanin, the propsed method validated by our group presents some advantages. The proposed method is fast, simple, accessible and unpublished for the quantification of this antibiotic.

The detection wavelength was set at 277 and 281 nm and the percentage of methanol in the mobile phase applied in the ratios of 52 : 48 (v/v) and 48 : 52 of methanol : acetonitrile. The results obtained from assays of the test solutions were not affected by varying the conditions and were in accordance with the results for original conditions. The %RSD value of assays determined for the same sample under original conditions and robustness conditions was less than 2.0% indicating that the developed method was robust.

We could conclude that the chromatographic method proposed is simple, rapid with low reagents cost and can therefore be applied for the determination of teicoplanin in lyophilized powder for injection. Method validation showed good results and included precision and accuracy.

This HPLC method proposed, fully validated, can therefore be applied for the determination of teicoplanin powder for injection.

4. Conclusions

In this study, a sensitive, precise, accurate, fast and specific RP-HPLC for quantification of teicoplanin in lyophilized preparations was developed and fully validated. All parameters meet the acceptance criteria for method validation according to the FDA and ICH specifications and the method shows range, linearity, precision, accuracy, specificity and recovery. It is also found that the excipients in the commercial lyophilized preparation did not interfere with the assay. The results showed the proposed method is suitable for its intended use. The method uses simple reagents, with minimum sample preparation procedures and short analysis time, encouraging its application in routine analysis. The results indicated that the proposed method might be recommended in routine quality control of teicoplanin powder for injection.

Acknowledgements

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