

Flow injection analysis of vancomycin

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Abstract: A flow injection method for the quantitative analysis of vancomycin hydrochloride, C₆₆H₇₅Cl₂N₉O₂₄.HCl (HVCM), based on the reaction with copper (II) ions, is presented. HVCM forms a lilac-blue complex with copper ions at pH≈4.5 in aqueous solutions, with maximum absorption at 555 nm. The detection limit was estimated to be about 8.5×10⁻⁵ mol L⁻¹; the quantitation limit is about 2.5×10⁻⁴ mol L⁻¹ and about 30 determinations can be performed in an hour. The accuracy of the method was tested through recovery procedures in presence of four different excipients, in the proportion 1:1 w/w. The results were compared with those obtained with the batch spectrophotometric and with the HPLC methods. Statistical comparison was done using the Student's procedure. Complete agreement was found at a 0.95 significance level between the proposed flow injection and the batch spectrophotometric methods, which present similar precision (RSD: 2.1 % vs. 1.9%).

Keywords: vancomycin; flow injection analysis; spectrophotometry; copper (II) ions.

Introduction

Vancomycin is a glycopeptidic antibiotic very efficient against a number of gram positive microorganisms, inhibiting the synthesis of the bacterial wall [1,2]. It is produced by the fermentation of the Actinobacteria species *Amycolatopsis orientalis* formerly *Nocardia orientalis* [3]. Its molecule shows a complex tricycle structure containing amino acids and sugars [4,5]. It was discovered in 1956 and its use in medicine began in 1959, after researches developed by the Laboratories Lilly in the United States [5]. Problems with vancomycin use, resulting in the clinical treatments via parenteral, are related to its nephrotoxicity, neurotoxicity and auditive damages [6].

The use of vancomycin was inhibited in the years 1960 and 1970 as the discovering of semi-synthetic varieties of penicillin which were

presenting smaller side effects. With the appearance of resistant microorganisms in the years 1980, and with the improvement of the processes of preparing and purifying, vancomycin acquired again an important role in the combat to the systemic staphylococcal infections in allergic patients to penicillin and in infections caused by staphylococci resistant to methicillin. It presents efficacy, administered orally, to the combat of *Clostridium difficile*, responsible for colitis that resulted from the use of antibiotics [1,2,7,8].

There are, in the literature, several methods for the determination of vancomycin in biological fluids and in pharmaceutical products [9], however only few spectrophotometric methods are reported for the analysis of vancomycin. Recently we proposed a green and simple spectrophotometric batch procedure based in the reaction of this antibiotic with ions copper (II) in

ethanol/water solution [10]. In this opportunity we are reporting a green flow injection analysis (FIA) procedure where it was used the same reaction of the antibiotic with copper, but now in aqueous solution. As it is known, FIA methods present some interesting qualities as, for instance, high analytical frequency, low cost and use of small aliquots [11].

Experimental

Materials

All the reagents used in this work were of analytical grade except the vancomycin hydrochloride, a pharmaceutical certificated product with 96.8 % purity, gently furnished by the Laboratório Antibióticos do Brasil Ltda. Distilled water was obtained from a glass distiller device.

Solutions

Vancomycin solutions: were daily prepared dissolving 0.7670 g of the 96.8 % purity vancomycin hydrochloride in 50 mL of distilled water, in order to obtain a 1.00×10^{-2} mol L⁻¹ concentration. From this solution, by appropriate dilutions, the other solutions of the antibiotic were prepared.

Copper acetate: 0.5989 g of Cu(CH₃COO)₂·H₂O in 100.0 mL of water in order to obtain a 3.00×10^{-2} mol L⁻¹ solution.

Influence of excipients

Despite the fact that vancomycin is more used in the pure form, in order to test the recovery from samples, it was studied the influence of four common excipients, in the proposed method. Aliquots of 1.0 g of vancomycin were separately mixed with 1.0 g of each one of the following excipients: starch, cellulose, lactose or silica. These mixtures were carefully triturated and homogenized in a mortar and then kept in closed glass flasks.

The sample containing lactose, as this excipient is soluble, was dissolved directly in water. Aliquots of about 145 mg, exactly weighed to 0.1 mg, were dissolved and diluted to 10.0 mL in a volumetric flask. In the cases of the non soluble (silica and cellulose) or partially soluble (starch), the aliquot was dissolved in 2 mL of water in a glass test tube, following vigorous manual shaking dur-

ing 1 minute and centrifugation for 3 minutes at 3000 rpm. The supernatant was separated with the aid of a pipette and put in a 10.0 mL volumetric flask. This operation was repeated twice. The volume in the volumetric flask was completed with water to the mark. These solutions presented a concentration of about 5×10^{-3} mol L⁻¹ of vancomycin hydrochloride and they were used for the proposed method and HPLC method [12].

Apparatus

Spectrophotometers-UV-Vis Pharmacia Biotech Ultrospec 2000 to obtain the spectra. Femto model 600S to perform the analyses.

Cells - All the measurements were made with Hellma glass cells of 1.000 cm of path.

HPLC - Waters 600 E with UV-Vis Waters 484 detector.

pHmeter - Analyser pH 300.

Centrifuge - Fanem Excelsa II Model 206 BL.

Peristaltic pump - Cole Parmer Masterflex L/S model 7519-25.

Magnetic stirrer - IKA model tab disc.

Magnetic mixer - Previously described [13].

Potentiometric recorder - Linear model 92425.

Sample valve - Previously described [14].

Polyethylene tubes - 1 mm i.d.

Tygon® tubes - (1.75 mm i.d. - Ismatec Two - Stop / Blue- Gren).

Methods

Flow injection

The method is based on the spectrophotometric detection of a colored complex formed between vancomycin and ions copper (II) [15]. The flow injection manifold is depicted in figure 1. The aqueous solutions of vancomycin (pH ≈ 3.5) were mixed with the reagent solution, copper (II) acetate aqueous solution (pH ≈ 6.0), in the mixer with magnetic stirring. The resulting solution, containing the complex, presents a pH ≈ 4.5.

Chromatographic (HPLC) reference method

As reference method was used the HPLC procedure described by Lunn [12] which is equivalent to that recommended by the United States Pharmacopeia [16].

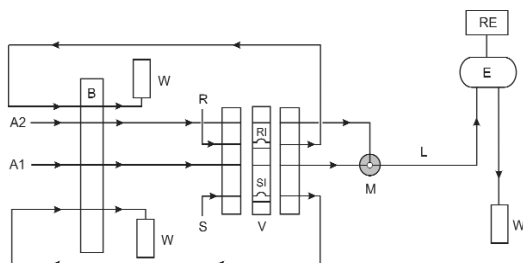


Figure 1. Scheme of the flow manifold used. **A1** = flow of water; **A2** = flow of water; **R** = introduction of the copper (II) solution $3.0 \times 10^{-2} \text{ mol L}^{-1}$; **S** = introduction of vancomycin solution $1.0 \times 10^{-2} \text{ mol L}^{-1}$; Flow rate **A1** = flow rate **A2**; **B** = peristaltic pump; **W** = waste; **RI** = reagent injection loop; **SI** = sample injection loop; **V** = sampling valve [4,5]; **M** = mixer with stirring; **L** = reaction bobbin (50 cm); **E** = spectrophotometer; **RE** = chart recorder.

HPLC experiments were performed on a HPLC Waters 600E with UV-VIS Waters 484 detector operated at 280 nm, injector Rheodyne and interface Waters 746. The column used was a C_{18} Nova Park® 25 cm \times 4.6 mm i.d. The mobile phase, consisted of acetonitrile: triethylamine: buffer (7:1:92), was pumped at a flow-rate of 1 mL min^{-1} . The buffer was prepared with 4 mL of triethylamine in 2 liters of water; the pH was adjusted to 3.2 with phosphoric acid. The calibration curve was constructed between $1.0 \times 10^{-5} \text{ mol L}^{-1}$ and $5.0 \times 10^{-4} \text{ mol L}^{-1}$ of vancomycin.

Results and discussion

The method is based on the measurement of the absorbance of the complex formed between vancomycin and copper (II) ions. The maximum absorption occurs at 555 nm [10]. For this work 525 nm was selected to monitor the signal in order to avoid the small absorption of the copper (II) acetate at 555 nm. The adoption of this wavelength means a decrease of about 10% in the absorbance signal fact that is counterbalanced by the absence of the small interference by the residual copper (II) acetate solution.

The optimization of the molar proportion copper: vancomycin was studied and the minimal relation 3:1 was found to be adequate as it

was observed an enough high analytical signal with none interference from the excess of copper (II) acetate. Therefore this relation was adopted in this work.

It was used ethanol-water solution in the batch method [10] in order to avoid the precipitation of the copper-vancomycin complex and to increase its absorbance in the visible region of the spectrum (555 nm). In the present case, however, as the flow method conducts the complex to the detector immediately after the reaction, no precipitation was observed. Also, the molar absorbance in water solution is quite enough for the analysis of pharmaceutical products. Therefore, in order to obtain a greener method even ethanol was avoided. In the batch method it was also necessary to adjust the pH at about 4.5 with acetic acid/acetate buffer in order to optimize the reaction mainly in the case of vancomycin base. In the present case however, working only with the main form of vancomycin, i.e., the hydrochloride (HVCM) and with copper (II) acetate, the final pH falls in the optimal region, between 4 and 5. Therefore, the buffer was not added in for minimizing the use of reagents.

Parameters as flow rate, aliquot volume and volume of the copper (II) acetate solution were also studied. As it was used a mixer with magnetic stirring that causes complete mixing of the solutions and increases the residence time due to its volume (113 μL), the length of the reaction bobbin showed to be not important and 50 cm was the size chosen for the connection to the flow cell in the spectrophotometer. Equal flow rates were used for the two lines, one for the aliquot and the other for the reagent.

The influence of the total flow rate was studied from 1.3 mL min^{-1} to 3.0 mL min^{-1} . Only a small decrease of the signal occurred in going from 1.3 mL min^{-1} to 2.0 mL min^{-1} . After this flow rate a rapid decrease was observed. Therefore the flow rate of 2.0 mL min^{-1} was adopted for this work, combining signal intensity with analytical frequency.

The aliquot volume was studied from 50 μL to 250 μL using always 250 μL of the reagent solution. It was observed an important increase of the signal up to 150 μL . With 200 μL only an increase of 1% was observed, the signal remaining of the

same intensity beyond this volume. Therefore, for the aliquots, 150 μL was used in this work.

The influence of the volume of the reagent solution was studied from 50 μL to 250 μL . As expected, an important increase of the signal was observed with the rise of the volume of the reagent but only up to 200 μL . With 250 μL only a small increase of about 2% occurred. The volume of 200 μL was adopted.

Therefore, the following conditions were used in the flow system: total flow rate 2.0 mL min^{-1} ; aliquot volume 150 μL ; reagent solution volume 200 μL ; length of the connection tube from the mixer to the spectrophotometric cell 50 cm; minimal molar proportion of the solutions copper (II) vancomycin 3:1. The experiments were all done at the room temperature (mean of 25 $^{\circ}\text{C}$).

The calibration curve was obtained using vancomycin hydrochloride solutions from $1.0 \times 10^{-3} \text{ mol L}^{-1}$ to $5.0 \times 10^{-3} \text{ mol L}^{-1}$. The calibration curve is described by the equation $h=0.50+3532C$, ($R= 0.995$) where h is the signal height in centimeters (1 cm = 0.8 mV) and C the antibiotic concentration.

The detection limit was estimated to be about $8.5 \times 10^{-5} \text{ mol L}^{-1}$; the quantitation limit is about $2.5 \times 10^{-4} \text{ mol L}^{-1}$.

Usually vancomycin hydrochloride is applied in the pure form, therefore, pharmaceutical preparations of this antibiotic are not common. In order to check the performance of the proposed method with respect to the recovery in presence of excipients, it was tested in presence of four different excipients; lactose, starch, silica and cellulose in the proportion 1:1 w/w. The results of those recovery experiments are shown in table 1 together with the results obtained with the batch [10] and with the HPLC method [12]. In the three methods the extraction of vancomycin from the sample was performed in the same way, i.e., according to the procedure described above in the experimental section. In the HPLC method, however, those solutions were diluted in order to obtain a final concentration of about $1.0 \times 10^{-4} \text{ mol L}^{-1}$, adequate to be introduced in the chromatograph. From this point each method was performed using the specific procedure.

Table 1. Vancomycin hydrochloride recovery from samples containing pharmaceutical excipients. Comparison between the results obtained with the proposed, with the batch [10] and with the HPLC method [12] using the statistical Student's t test and the **F** test.

Excipient	Proposed ^a \pm SD %	Batch method ^a \pm SD %	Calculated t^c	Calculated F^d	HPLC ^b \pm SD %	Calculated t^c	Calculated F^d
Starch	98.2 \pm 1.4	99.2 \pm 0.6	0.93	5.4	99.8 \pm 2.3	0.84	2.7
Cellulose	102.6 \pm 0.9	103.7 \pm 0.2	1.69	20.3	98.0 \pm 1.3	4.11	2.1
Silica	98.1 \pm 1.8	100.4 \pm 1.3	1.46	1.9	109.2 \pm 3.8	3.73	4.5
Lactose	98.3 \pm 1.3	98.7 \pm 0.2	0.43	42.3	93.7 \pm 1.8	2.93	1.9
Mean ^e	99.3 \pm 2.1 n=12	100.4 \pm 1.9 n=12	1.29	1.27	100.2 \pm 6.1 n=12	0.46	8.4

^aProposed and batch methods: HVCM concentrations in the final solution before reacting with copper (II) were $5.0 \times 10^{-3} \text{ mol L}^{-1}$.

^bHPLC method: HVCM concentration after extraction was $2.1 \times 10^{-3} \text{ mol L}^{-1}$; this solution was diluted to $1.0 \times 10^{-4} \text{ mol L}^{-1}$ to be injected in the chromatograph.

^cTheoretical Student's $t = 2.78$ ($\alpha=0.05$) and 4.60 ($\alpha=0.01$) for the degree of freedom $v=4$ ($v= n_1 + n_2 - 2$); $n_1 = n_2 = 3$.

^dTheoretical $F = 19.00$ ($\alpha=0.05$) and $F = 99.00$ ($\alpha=0.01$) ($v_1=v_2=2$) [17].

^eFor ($v_1=v_2=11$; $n_1=n_2=12$) $F = 2.87$ ($\alpha=0.05$) and $F = 4.44$ ($\alpha=0.01$). Theoretical Student's $t = 2.07$ ($\alpha=0.05$) and 2.81 ($\alpha=0.01$) for the degree of freedom $v= 22$ ($v= n_1 + n_2 - 2$); $n_1 = n_2 = 12$. The experimental analytical data were statistically treated as a whole, independently of the present excipient.

In table 1, in order to give an idea of the average result, independently of the used excipient, the means of the recoveries were calculated for the three methods and for the four different excipients. Comparing the results obtained with the proposed method with that of the batch method, based on the Student's *t* test [17], they can be considered statistically equal for the 0.95 ($\alpha=0.05$) significance level. The **F** test shows a small difference with respect to the precision for $\alpha=0.05$ for the samples containing cellulose and lactose but, in the 0.99 ($\alpha=0.01$) significance level there is concordance in all cases. For the means, complete concordance is observed for the 0.95 significance level.

Comparing the results obtained with the flow method with those obtained with HPLC, based on the Student's *t* values only in the case of starch is observed concordance for the 0.95 ($\alpha=0.05$) significance level. The **F** test shows agreement between the methods in terms of precision. For $\alpha=0.01$ both Student's *t* and **F** test show that there is statistical equivalence between the two methods.

If it is considered that a tolerance of 5% in the precision of the quantitative analysis of vancomycin is allowed (18), complete coherence among the three methods is observed.

In order to test the specificity of the method [19] some aliquots (*ca.* 75 mg) of the samples were submitted to stress conditions with heating: base (0.1 mol L⁻¹ NaOH); oxidant (3% H₂O₂ w/v); acid (0.1 mol L⁻¹ HCl); water. Vancomycin chloridrate was dissolved in the respective medium and the obtained solutions were heated to 80 °C for 30 minutes. After this treatment the obtained solution was mixed with copper (II) acetate solution according to the

explained above in samples treatment. In all cases no reaction was observed.

Conclusion

The proposed method, applied to pure vancomycin hydrochloride or in presence of starch, lactose, cellulose or silica, offers analytical quantitative results of good precision and accuracy; it can be considered a green procedure as it avoids the use of organic solvent and of potentially toxic reagents; the used volumes are relatively small; it is very simple to be performed; the instrumentation used is of relatively low cost; the results can be obtained in about 3 minutes for the bulk antibiotic (in comparison with 20 minutes in HPLC method), including the dissolution of the aliquot, or in about 9 minutes, including the extraction procedure in presence of the excipients; in the flow system about 30 determinations can be performed in an hour. Based on these results the method can be recommended for the quantitative analysis of HVCM in the conditions presented in this article.

Acknowledgments

The authors are grateful to CAPES, Coordenação de Aperfeiçoamento de Pessoal de Nível superior, to CNPq, Conselho Nacional de Pesquisa Científica e Tecnológica, and to FAPESP, Fundação de Amparo à Pesquisa do Estado de São Paulo, for the financial support.

Received 14 May 2008

Accepted 11 June 2008

Resumo: Este trabalho apresenta um método de análise por injeção em fluxo para determinação quantitativa de cloridrato de vancomicina, $C_{66}H_{75}Cl_2N_9O_{24}\cdot HCl$ (HVCM), baseado na reação com íons cobre (II). HVCM na presença de íons cobre, em $pH=4,5$ e solução aquosa forma um complexo azul-lilás, com absorção máxima em 555 nm. O limite de detecção estimado foi de $8.5 \times 10^{-5} \text{ mol L}^{-1}$; e o limite de quantificação de $2.5 \times 10^{-4} \text{ mol L}^{-1}$ com cerca de 30 determinações por hora. A exatidão do método foi determinada através de ensaio de recuperação na presença de quatro diferentes excipientes na proporção de 1:1 p/p. Os resultados do método espectrofotométrico por injeção em fluxo foram comparados com resultados obtidos pelo método espectrofotométrico por batelada e por HPLC, através de método estatístico t de Student. Obteve-se completa concordância com nível de significância de 0,95 e similar precisão (RSD: 2,1 % vs. 1,9%).

Palavras-chave: vancomicina; análise por injeção em fluxo; espectrofotometria; íons de cobre II.

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