

STABILITY-INDICATING HPLC METHOD FOR DETERMINATION OF VANCOMYCIN HYDROCHLORIDE IN THE PHARMACEUTICAL DOSAGE FORMS

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Abstract: A simple, rapid and selective RP-HPLC method was developed for the determination of vancomycin hydrochloride. The separation was achieved using a Capital C8-Optimal column (250 × 4.6 mm i.d., 5 μm particle size) with a mobile phase composed of buffer citrate (pH 4), acetonitrile and methanol in the ratio of 85 : 10 : 5 (by volume), respectively. The mobile phase was pumped using an isocratic HPLC system at a flow rate of 1 mL/min and quantification of analyte was based on measuring its peak areas at 280 nm. Cephalixin monohydrate was used as internal standard (IS). The retention times for vancomycin hydrochloride and cephalixin were about 4.30 and 7.50, respectively. The reliability of the proposed HPLC procedure was validated with respect to linearity, ranges, precision, accuracy, specificity and detection limit. Calibration curve was linear in the ranges of 1-100 μg/mL with correlation coefficient of 0.9999. The proposed method proved to be selective and stability-indicating by the resolution of the analytes from the forced degradation (hydrolysis, oxidation, thermolysis and photolysis) products. The validated HPLC method was successfully applied to the analysis of vancomycin hydrochloride in pharmaceutical dosage forms. The degradation products resulted from the storage of the drug under stress degradation conditions described by the International Conference on Harmonisation (ICH).

Keywords: vancomycin hydrochloride, RP-HPLC, stability-indicating, forced degradation, internal standard

Vancomycin (Fig. 1) is an amphoteric glycopeptide antibiotic, first isolated in 1956, that is active against a broad range of Gram positive bacteria as well as some Gram negative cocci. It has a strong bactericidal activity, inhibiting the cell wall synthesis. Because of toxicity, vancomycin was used primarily for alternative therapy before the recent emergence of methicillin-resistant and penicillin-resistant organism (1, 2). For patients allergic to both the penicillins and cephalosporins, vancomycin is often the only effective treatment (1, 2). The United States Pharmacopeia and the National Formulary (USP-NF) method for the quantitation of vancomycin is based on microbiological assay (3). Due to its increased clinical significance, a number of high performance liquid chromatographic meth-

ods for the determination of vancomycin in biological fluids have been developed (4-9).

Due to the vital significance of vancomycin, the development of a simple and fast method for its quantification is of significant need. The aim of the present work was to develop a simple, accurate, and precise stability-indicating HPLC method to evaluate vancomycin in the presence of its degradation products in the pharmaceutical dosage forms.

MATERIALS AND METHODS

Chemicals

Working standards of vancomycin and cephalixin monohydrate were purchased from Hebbi union pharmaceutical company. HPLC grade

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solvents and reagents were purchased from Merck (Germany).

HPLC instrumentation

The HPLC system consisted of a Knauer Smartline pump 1000 and a Smartline UV detector. The column was Optimal® C8 (250 × 4.6 mm i.d., 5 µm particle size). Chromgate software was used for data acquisition. Sample injection was done by Rheodyne manual injector.

Chromatographic conditions and mobile phase preparation

Chromatographic separation was achieved using Optimal C8 column that was purchased from Capital, U. K. The HPLC system was operated isocratically using a mobile phase consisting of buffer citrate (pH 4) : acetonitrile : methanol in the ratio of 85 : 10 : 5 (v/v/v) as the mobile phase at a flow rate of 1.0 mL/min at room temperature. The mobile phase was filtered using a 0.45 µm microporous filter and was degassed prior to use. The injection volume was 100 µL. The UV detector was set at 280 nm. The peak areas were integrated automatically using Chromgate software. The total run time of the system was about 10 min.

Different combinations of buffers and organic solvents were tested to obtain optimum separation. Finally a mobile phase composed of citrate buffer,

acetonitrile and methanol (85 : 10 : 5, v/v/v) was chosen to separate vancomycin, (using cephalixin as internal standard) and the degradation products.

All buffer solutions were adjusted to acidic pH due to low stability of vancomycin at alkaline pH and it was found that the pH of maximum stability for vancomycin is between 3.0 and 5.0 (7).

Selection of suitable internal standard

Different compounds were tested in order to determine the best internal standard (IS) for analysis of vancomycin hydrochloride. Using peak shape and retention time as an indicator, cephalixin monohydrate was chosen as internal standard in this experiment.

Preparation of standard solutions

A 1000 µg/mL stock vancomycin solution was prepared in deionized water. Standard vancomycin solutions (1-100 µg/mL) were obtained with serial dilutions. These standard solutions were used for the method validation. An equal amount of a 200 µg/mL cephalixin stock solution was added to the vancomycin standard solutions prior to the injection to the HPLC system.

Method validation

Method was validated in accordance to the ICH guidelines (11). Parameters such as linearity,

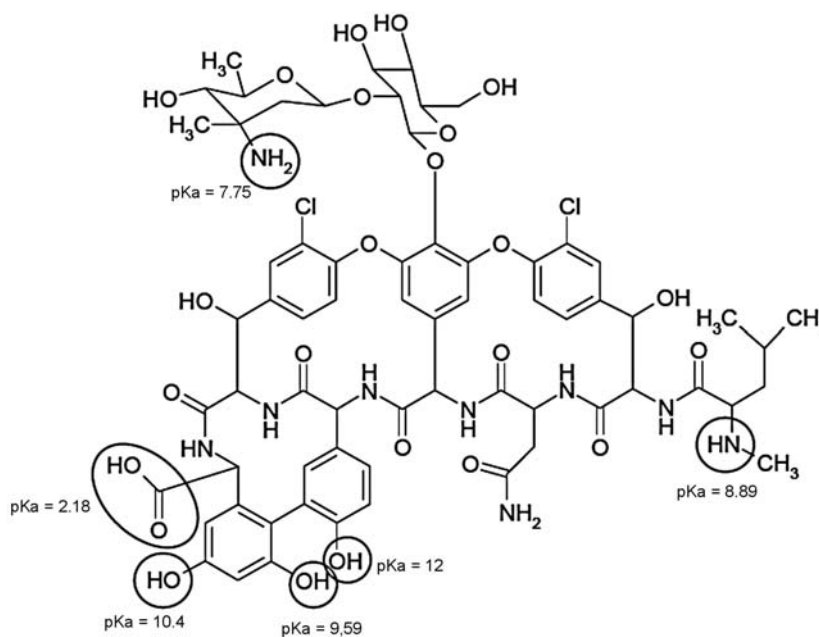


Figure 1. Structure of vancomycin hydrochloride

range, accuracy (recovery), precision (inter-day and intra-day precision), specificity and stability-indicating capability were all validated.

Linearity

For testing linearity seven calibration standards were prepared in the range of 1 to 100 µg/mL, (1, 5, 10, 25, 50, 75 and 100 µg/mL). Working standard solutions (1-100 µg/mL) of vancomycin were prepared by serial dilution in deionized water from 1 mg/mL stock solution. Calibration curve was plotted over the concentration range of 1-100 µg/mL for vancomycin hydrochloride using cephalixin as internal standard. Four hundred fifty µL of standard solutions were transferred to a series of 1 mL Eppendorf's tubes. Fifty µL of a 200 µg/mL cephalixin standard solution was added to each tube as internal standard prior to the injection to the HPLC system. The resulting solutions were then vortexed and injected into the column and the peak area obtained at retention times of about 4.30 and 7.50 min at a flow rate of 1 mL/min were measured at 280 nm for vancomycin and cephalixin, respectively. Calibration curve was constructed by plotting the ratio of peak area of vancomycin to the peak area of cephalixin (IS) *versus* concentration of vancomycin. Each reading was the average of three determinations.

Precision

To evaluate the intra-day (repeatability) and inter-day (reproducibility) precision, three replicates of standard solutions at three different concentrations in the range of calibration curve (other than calibration points) were assayed on the same day and on 3 different days, calculating the response (the ratio of vancomycin peak area to the ratio of cephalixin peak area) obtained and its coefficient of variation (CV%).

Accuracy (Recovery)

To check the accuracy of the method, five concentrations of calibration curve were analyzed; the concentrations were recalculated from the corresponding calibration straight line (experimental concentration) and were compared with the theoretical concentrations. Recovery was estimated as the relationship between the experimental concentration (C_{exp}) and the theoretical concentration (C_{theor}) expressed as percentage: $(C_{exp}/C_{theor}) \times 100$.

Sensitivity

Method sensitivity was indicated by limit of detection (LOD) and limit of quantification

(LOQ). LOD was established at a signal to noise ratio (S/N) of 3.3. LOQ was established at a signal to noise ratio (S/N) of 10. LOD and LOQ values were experimentally verified by 3 injections of vancomycin hydrochloride at the LOD concentration.

Specificity (placebo and forced degradation interference)

Specificity is the ability of a method to measure analytical response in the presence of its potential impurities. Specificity of the method was carried out by the deliberate degradation of the drug by oxidation, heat, hydrolysis (acidic, alkaline, and neutral) and photolysis followed by its analysis using the developed method.

The stress conditions studied were sunlight, heat (oven and bain-marie), acid hydrolysis (3.0 M HCl), base hydrolysis (1.5 M NaOH), and oxidation (3% H₂O₂). The sample stress solutions were analyzed against the freshly prepared standards.

Application of the method

The developed HPLC method was applied for determination of vancomycin hydrochloride in its pharmaceutical dosage forms. Vancomycin vial 500 mg/vial) was dissolved in 500 mL of distilled water and appropriate concentration of vancomycin solution was injected to the HPLC system using serial dilution method, adding cephalixin as internal standard prior to the injection. The process was repeated 3 times to evaluate the amount of drug in each vial. The dosage form then was affected with oxidative condition explained as follows.

Standard drug stock solutions

Forced degradation studies for the drug were carried out under the conditions of hydrolysis, oxidation, and photolysis. An accurately weighted quantity of the powder, 10 mg of vancomycin, was taken into a 10-mL measuring flask and diluted to the mark with deionized water to get a final concentration of 1000 µg/mL of vancomycin hydrochloride. The internal standard stock solution was prepared by dissolving 10 mg cephalixin in a 50-mL volumetric flask to get a final concentration of 200 µg/mL of cephalixin. These stock solutions were used for the forced degradation studies.

Acid hydrolysis

Forced degradation in acidic media was performed, in the first step, by preparing a 500 µg/mL solution of vancomycin from the stock solution using serial dilution method. Then, a concentration

of 50 µg/mL of vancomycin solution was prepared by adding 200 µL of HCl 3 M solution and 700 µL of deionized water to a 100 µL of 500 µg/mL vancomycin solution. This solution was vortexed at room temperature (RT) for 6 h and during this period, samples were injected to the HPLC system at different time intervals. Equal amounts of IS solution were added to the test solutions prior to the injection.

Base hydrolysis

Forced degradation in basic media was performed by, in the first step, preparing a 500 µg/mL solution of vancomycin from the stock solution using serial dilution method. Then, a concentration of 50 µg/mL of vancomycin solution was prepared by adding 200 µL of NaOH 1.5 M solution and 700

µL of deionized water to a 100 µL of 500 µg/mL vancomycin solution. This solution was vortexed at RT for 1 h and after this period samples were injected to the HPLC system. Equal amounts of IS solution were added to the test solutions prior to the injection.

Oxidative hydrolysis

Forced degradation in oxidative media was performed, in the first step, by preparing a 500 µg/mL solution of vancomycin from the stock solution using serial dilution method. Then, a concentration of 50 µg/mL of vancomycin solution was prepared by adding 200 µL of H₂O₂ 3% solution and 700 µL of deionized water to a 100 µL of 500 µg/mL vancomycin solution. This mixture was kept for up to 1 h in the dark and after this period samples

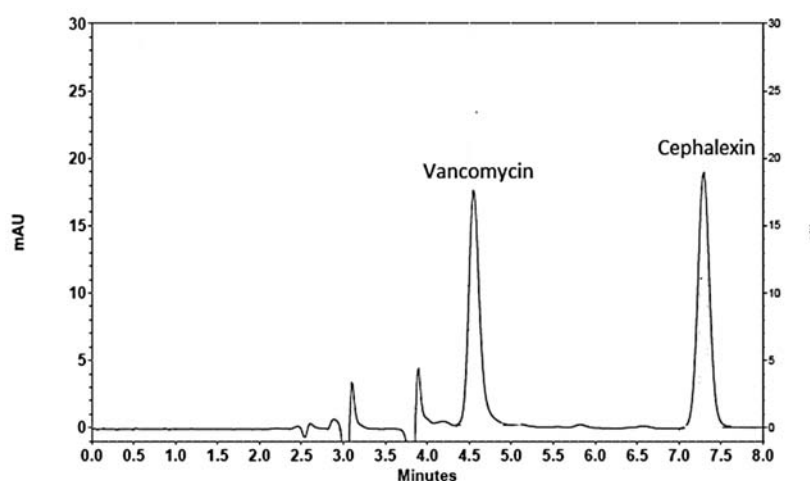


Figure 2: Typical chromatogram of vancomycin using cephalixin as IS

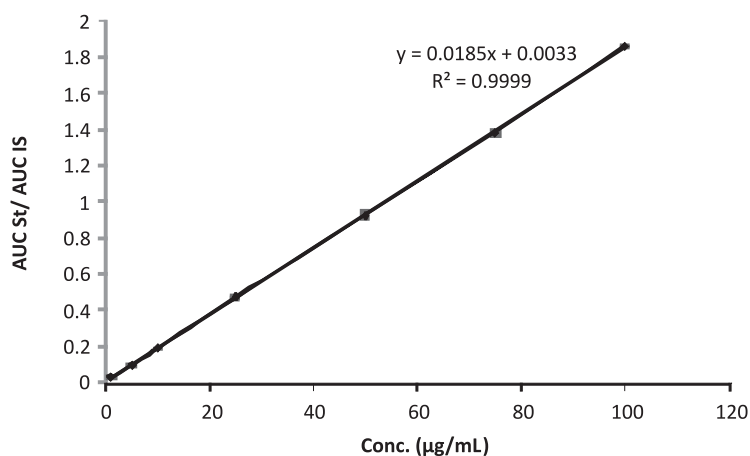


Figure 3: Calibration curve for vancomycin hydrochloride using cephalixin as IS

Table 1. Calibration curve standards of vancomycin hydrochloride.

Conc. ($\mu\text{g/mL}$)	Mean $\text{AUC}_{\text{st}}/\text{AUC}_{\text{is}} \pm \text{S.D.}$	CV% (RSD)
1	0.026 \pm 0.001	2.23
5	0.092 \pm 0.001	1.09
10	0.187 \pm 0.001	0.57
25	0.470 \pm 0.002	0.32
50	0.924 \pm 0.017	1.82
75	1.381 \pm 0.015	1.10
100	1.860 \pm 0.004	0.19

Table 2. Accuracy.

C theoretical	Accuracy %	Accuracy %	Accuracy %	Mean \pm S.D.	CV%
1	124.86	127.02	120.54	124.14 \pm 3.30	2.66
5	97.08	96.43	94.92	96.14 \pm 1.10	1.15
10	98.92	99.73	100.05	99.57 \pm 0.58	0.58
25	100.60	101.25	100.95	100.93 \pm 0.32	0.32
50	97.45	100.83	100.29	99.52 \pm 1.82	1.82
75	98.05	100.23	99.37	99.22 \pm 1.11	1.11
100	100.15	100.36	100.53	100.35 \pm 0.19	0.19

Table 3. Inter and intra-day precision.

Conc. ($\mu\text{g/mL}$)	Inter-day		Intra-day	
	Mean ($\text{AUC}_{\text{st}}/\text{AUC}_{\text{is}} \pm \text{S.D.}$)	CV%	Mean ($\text{AUC}_{\text{st}}/\text{AUC}_{\text{is}} \pm \text{S.D.}$)	CV%
8	0.149 \pm 0.004	2.42	0.146 \pm 0.003	1.88
40	0.764 \pm 0.013	1.73	0.754 \pm 0.004	0.59
80	1.494 \pm 0.054	3.64	1.450 \pm 0.034	4.21

were injected to the HPLC system. Equal amounts of IS solution were added to the test solutions prior to the injection.

Thermal degradation

To study thermal degradation, a 50 $\mu\text{g/mL}$ solutions of vancomycin was kept in oven (70°C) in order to study dry heat stability of vancomycin solution and another solution of drug was kept in benmarie (heath bath) (70°C) to evaluate drug stability in wet heat. In different time intervals samples were taken and injected to the HPLC system in order to monitor the drug degradation behavior. Equal amounts of IS solution were added to the test solutions prior to the injection.

Photodegradation

The photostability was studied by exposing the solid state of drug to direct sunlight in summer days for 6 h on a glass plate.

RESULTS AND DISCUSSION

From all the condition assayed, the best results were obtained with a mixture of 10 mM buffer citrate (pH 4) : acetonitrile : methanol (85 : 10 : 5, v/v/v) as the mobile phase at a flow rate of 1 mL/min.

Different compounds were tested for selection of IS such as cephalixin, cefixime, cephalozin, ampicillin, amoxicillin, caffeine, ondansetron and

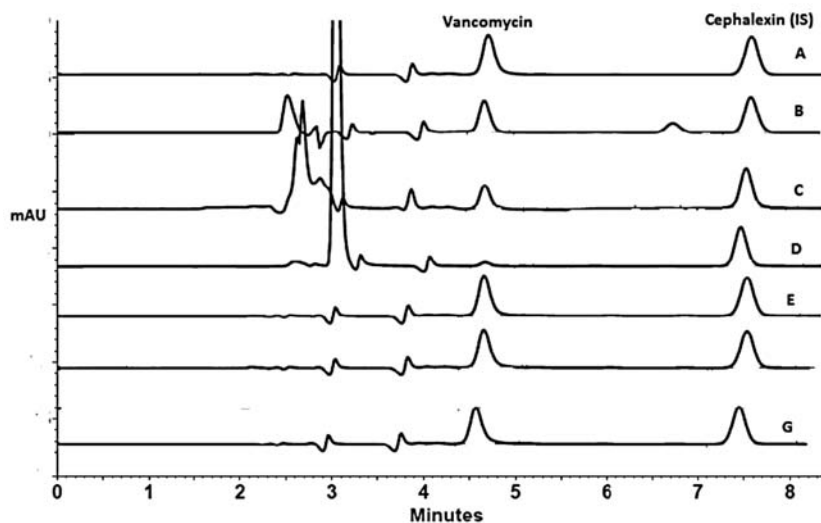


Figure 4. HPLC chromatograms of vancomycin under different stress conditions: A) vancomycin standard solution, B) acidic degradation (HCl 3 M) of vancomycin after 6 h, C) basic degradation (NaOH 1.5 M), D) oxidative degradation (H_2O_2 3%), E) dry heat (oven 70°C), F) wet heat (bain-marie 70°C), G) direct sun light

Table 4. Summary of the forced degradation of vancomycin hydrochloride.

Condition of forced degradation	Degradation % of vancomycin
3 M HCL, 6 h	25
1.5 M NaOH, 1 h	20
3% v/v H_2O_2 , 1 h	90
Oven 70°C , 5 h	No degradation
Bain marie 70°C , 5 h	No degradation
Sunlight (6 h, summer)	No degradation

teicoplanin. Among the tested compounds cephalixin was chosen as internal standard because its peak area (area under the curve: AUC) was comparable with vancomycin AUC in the same concentration range and its peak was eluted after vancomycin peak.

The representative chromatogram of vancomycin and cephalixin is illustrated in Figure 2.

Within the concentration range of 1-100 $\mu\text{g}/\text{mL}$, linearity was obtained with a correlation coefficient of 0.9999 (Fig. 3 and Table 1).

The sensitivity of the method was explored *via* measurement of the limit of detection (LOD). The LOD was found to be 10 ng/mL , with a RSD of less than 2%. Therefore, this method can be used to estimate very little amounts of drug even in biological studies.

Accuracy was determined using standard calibration curve of vancomycin within the range of 1-

100 $\mu\text{g}/\text{mL}$. In this range accuracy was calculated for concentrations of 1, 5, 10, 25, 50, 75 and 100 $\mu\text{g}/\text{mL}$. The data on accuracy are shown in Table 2, with coefficient of variations (CV) less than 3%, a value below the limit accepted by FDA (15%).

To evaluate the intra and inter-day precision, three replicates of standard solutions at three different concentrations (8, 40 and 80 $\mu\text{g}/\text{mL}$) were assayed on the same day and on three different days, calculating the response, the ratio of vancomycin area under the curve (AUC) to cephalixin AUC, and its coefficient of variation. Table 3 shows inter and intra-day precision.

The specificity of the HPLC method for vancomycin hydrochloride has been assessed by performing forced degradation studies on the drug to indicate stability of the drug to different conditions. The stress conditions studied were sunlight, heat (oven and bain-marie), acid hydrolysis (3.0 M HCl), base hydrolysis (1.5 M NaOH), and oxidation (3% H_2O_2). The sample stress solutions were analyzed against the freshly prepared standard. The different stress conditions and the assay results are summarized in Table 4.

Vancomycin showed extensive degradation under oxidative and mild under basic conditions of degradation. Resolution for both drug (vancomycin) and IS (cephalexin) was found to be greater than 2.0. Therefore, there was no interference between vancomycin and cephalixin peaks and any other degradation products peaks in the chromatogram. Figure 4 shows the chromatographic profiles of van-

comycin and the degradation products after exposing the prepared sample solution to different stress conditions as described in Table 4.

The developed HPLC method was applied for the determination of vancomycin in its pharmaceutical dosage forms. The results of triplicate analysis indicated that the amount of vancomycin was 495.2 ± 1.57 mg/vial which was in great accordance with the label claim (500 mg/vial).

CONCLUSION

The developed validated HPLC method for the quantitative determination of vancomycin hydrochloride, was evaluated for specificity, sensitivity, linearity, range, accuracy (recovery) and precision (intra-day and inter-day precision). All the validation results were within the allowed specifications of the ICH guidelines. The developed method has proven to be rapid, accurate, and stability-indicating for the determination of vancomycin hydrochloride in the presence of the degradation products. There was always a complete separation of both drug and internal standard from the degradation products. As a result, the proposed HPLC method could be adopted for the quantitative quality control and routine analysis of the vancomycin pharmaceutical dosage forms.

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Authors statement

The authors declare no conflict of interest.

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