

Stability Indicating HPLC Method for the Simultaneous Determination of Ceftriaxone and Vancomycin in Pharmaceutical Formulation

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Abstract

A simple, sensitive, rapid, robust and reproducible method for the simultaneous determination of ceftriaxone and vancomycin in formulation was developed using reverse phase high performance liquid chromatographic method. The analysis was performed on C8 (250×4.6 mm, 5 μm) column with a mobile phase consisting of 0.01 M of potassium di hydrogen ortho phosphate and 0.01M of disodium hydrogen phosphate buffer (pH 4.2), methanol in the ratio of 70:30 (v/v) with a flow rate of 1ml/min. The analyte was examined with UV detector at 298 nm. In the developed method vancomycin elutes at 2.7 min and ceftriaxone at 3.7 min. The proposed method is having linearity in the concentration range from 20-100 μg/ml of ceftriaxone and 10-50 μg/ml vancomycin. The method was validated with respect to system suitability, precision, linearity, limit of detection (LOD) and limit of quantification (LOQ), accuracy, recovery, robustness, stability. The proposed method can be readily utilized for determination of ceftriaxone and vancomycin.

Keywords: Ceftriaxone; Vancomycin; RP-HPLC; Stability indicating method; Pharmaceutical formulation

Introduction

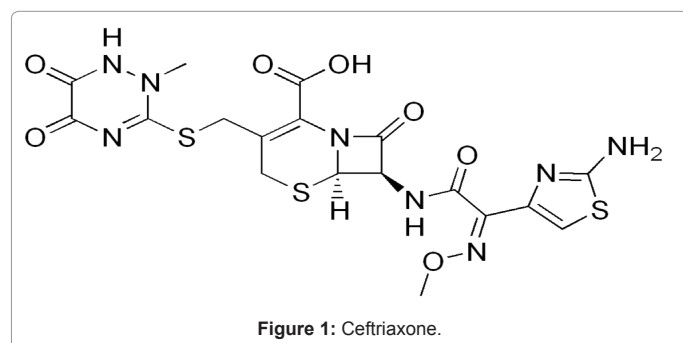
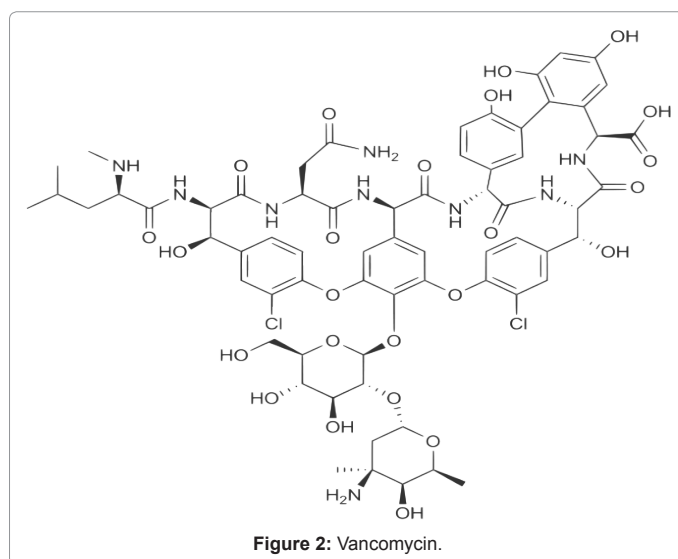
Ceftriaxone is (6R,7R)- 3 [(acetyl - oxy) methyl] -7-[[[(2Z)-(2-amino-4 thiazolyl) (methoxyamino)-acetyl]amino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (Figure 1).

Ceftriaxone is a third generation cephalosporin beta-lactam antibiotics used in the treatment of bacterial infections caused by vulnerable, generally gram positive organism. Bactericidal activity of ceftriaxone is due to inhibition of the cell wall synthesis and is facilitated by ceftriaxone binding to penicillin-binding proteins. It hinders the mucopeptide synthesis in the bacterial cell wall. The beta lactam moiety of the ceftriaxone binds to carboxypeptidase, endopeptidase, and transpeptidase in the bacterial cytoplasmic membrane, which are responsible for cell wall synthesis and cell division. Ceftriaxone results in the formation of defective cell walls and cell death by binding to these enzymes [1].

The bactericidal action of vancomycin (Figure 2) is due to inhibition of cell-wall biosynthesis. Specifically, vancomycin avoids integration of N-acetylmuramic acid (NAM) and N-acetyl glucosamine (NAG)-peptide subunits from getting incorporated into the peptidoglycan matrix; which is the major structural component of Gram-positive cell walls. The huge hydrophilic molecule is capable to form hydrogen bond interactions with the terminal D-alanyl-D-alanine moieties of the NAM/NAG-peptides. Usually this is a five-point interaction. The binding of vancomycin to the D-Ala-D-Ala stops the incorporation of

the NAM/NAG-peptide subunits into the peptidoglycan matrix. In addition, vancomycin varies bacterial-cell-membrane permeability and RNA synthesis and there is no cross-resistance between vancomycin and other antibiotics. So vancomycin is not active in vitro against gram-negative bacilli, fungi, or mycobacteria [2,3].

Vancopluse (ceftriaxone-vancomycin) is a synergetic antimicrobial



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combination with marked in vitro antibacterial activities against broad spectrum gram-negative and gram-positive bacteria. Since ceftriaxone is less active against gram-positive cocci than first generation cephalosporins, marked activities are seen against gram-negative bacteria such as Enterobacteriaceae including beta-lactamase producing strains. It is active against *S. aureus* but not against methicillin resistant *S. aureus*. However vancomycin acts against a variety of gram-positive bacteria including MRSA and *S. epidermidis*. By combining these two drugs a fixed dose combination developed covers wide range of gram-positive and gram-negative bacteria. The combination is made compatible by adding a chemical vector which further reduces toxic effects.

The synergistic action of ceftriaxone-vancomycin has strong bactericidal activity against *S. aureus* and methicillin resistant strains. The combination works well against enterococci, prevents development of resistance, and has been used successfully against a wide range of bacterial infections including bacterial meningitis.

A literature survey revealed there are several methods to estimate ceftriaxone, including high performance liquid chromatography (HPLC) [4-8], high performance thin layer chromatography [9], capillary electrophoresis [10,11] and spectrophotometry [12-14]. In case of vancomycin, it has been assayed by HPLC [15,16], micellar electro kinetic capillary chromatography [17], and spectrophotometry [18].

However, there is only one method for the simultaneous estimation of ceftriaxone and vancomycin by HPLC [19] with high linearity values and difficult to detect in lower concentration. The stability studies of the combined drugs are not reported in the determination of drugs. The aim of the present study was to develop a simple, sensitive, accurate, versatile, and fast stability indicating HPLC method for the simultaneous estimation of ceftriaxone and vancomycin in pharmaceutical injection dosage form.

Materials and Methods

Chemicals and reagents

Pure sample of ceftriaxone and vancomycin was received from Strides Arco Labs, Bangalore. Formulation of ceftriaxone and vancomycin namely VANCOPULSE was obtained from local pharmacy product was labeled to contain 1 mg of ceftriaxone and 0.5 mg of vancomycin. HPLC grade water, HPLC grade methanol were procured from Merck Pvt Ltd., Mumbai, India. All other chemicals used are analytical reagent grade (AR grade) like potassium dihydrogen orthophosphate and orthophosphoric acid and sodium dihydrogen phosphate procured from Loba Chemie, Mumbai.

Instrumentation

A HPLC equipped with UV detector was used for the present research work. The separation was achieved using C-8 column. The mobile phase was a mixture of phosphate Buffer of (pH-4.2), and methanol (70:30) v/v. The contents of mobile phase was filtered before use through 0.45 μ membrane filter and its was degassed with a helium sparge for 15 min at flow rate of 1.0 ml/min. Here the column temperature was maintained at $20 \pm 10^\circ\text{C}$. The injection volume of samples was 10 μ l and the analyte was monitored at wavelength of 298 nm. The chromatographic conditions are shown in Table 1.

Method Development

Taking in attention the instability of ceftriaxone and vancomycin

Parameter	Optimized condition
Chromatograph	HPLC with UV-detector
Column	C8 Column
Mobile Phase	pH-4.2 phosphate buffer in the ratio and methanol of 70:30 (v/v)
Flow rate	1.00 ml/min
Detection	298 nm
Injection volume	10 μ l.
Temperature column	Room temperature

Table 1: Optimized chromatographic conditions.

in the strong alkaline and strong acidic conditions and pH value of the mobile phase should be limited within the range of 3-7. As mild acidic pH favors the retention and separation of two drugs on C-8 column. After some trials phosphate buffer with pH 4.2 was finally selected. The method development started with the methanol and phosphate buffer. In this mobile phase, there solution of the two drugs was not acceptable, so to change the selectivity the organic phase was changed from methanol to acetonitrile. Since both ceftriaxone and vancomycin in the mobile phase have no significant UV maximum but has end absorption, so to ensure the sensitivity of the method, the wavelength of 298 nm was employed for the detection. After a number of preliminary experiments, a Phenominax C-8 column and binary mixture of phosphate buffer pH 4.2 and methanol (70:30% v/v) was optimized as the mobile phase which produced good resolution, symmetric peak shape, and reasonable retention time for both the drugs. The retention times of ceftriaxone and vancomycin for six repetitions were found to be 3.7 ± 0.02 min and 2.7 ± 0.006 respectively. The chromatogram of a standard and sample solution is shown in Figure 3.

Preparation of mobile phase

The content of the mobile phase was prepared from filtered and degassed mixture of 3.5 g Potassium dihydrogen orthophosphate and 14.5 g of disodium hydrogen phosphate is taken in 1000 ml flask and made up to mark with water and pH is adjusted to (pH 4.2) with ortho phosphoric acid and methanol in the ratio of 70:30 v/v.

Preparation of standard solutions

About 100 mg of pure sample of ceftriaxone and 100 mg vancomycin was accurately weighed and dissolved in HPLC grade water in a 100 ml standard flask separately to get a standard stock solution concentration of 1 mg/ml (1000).

Preparation of working standard solutions

Working standard solutions (20-100 μ g/ml) of ceftriaxone and (10-50 μ g/ml) vancomycin were prepared by from standard solution with HPLC graded water. These working solutions were used for preparation of plasma samples, validation and quality control samples.

Preparation of calibration curve

Aliquots of (20-100 μ g/ml) of ceftriaxone and (10-50 μ g/ml) vancomycin were prepared into a series of 10 ml volumetric flask from 1000 μ g/ml of stock solution. The volume was made up to the mark with mobile phase, all standard solutions are injected and measured at 289 nm. The calibration curve was established by plotting the peak areas of ceftriaxone and vancomycin versus the concentrations of ceftriaxone and vancomycin.

Preparation of sample solution of formulation

VANCOPULSE (ceftriaxone-vancomycin) containing 1000 mg of ceftriaxone and 500 mg vancomycin was taken and the weight was

calculated. Powdered sample equivalent to 100 mg of ceftriaxone and vancomycin which was taken and transferred to 100 ml volumetric flask and made up to the mark (final concentration equal to 1000 µg/ml) with water. The sample was mixed well and filtered through 0.45 µm membrane filter then clear filtrate was diluted to desired concentrations.

Assay procedure

The column was equilibrated for minimum 30 min, with the mobile phase flowing through the system with a flow rate of 1ml/min. Detector was set at a wavelength of 298 nm. Five sets of the drug solutions were prepared having 10-50 µg/ml for vancomycin and 20-100 µg/ml ceftriaxone in mobile phase mixture. The retention time of vancomycin and ceftriaxone in bulk drug in five replicate samples were found to be 2.7 min and 3.7 min (Figure 3) and the retention time of vancomycin and ceftriaxone in its pharmaceutical formulation were found to be 2.7 min and 3.7 min (Figure 4) it is done by taking 40 µg/ml of VANCOPLUSE which contains (40 µg/ml of ceftriaxone and 20 µg/ml vancomycin) respectively and the peak areas of the drug concentration were calculated. Ultimately we end up in regression of the drug concentration over the peak areas.

Results and Discussion

Method validation

The method was validated for different parameters like linearity, accuracy, precision, robustness, limit of detection (LOD) and limit of quantification (LOQ) [20].

System suitability

System performance parameters of the developed HPLC method

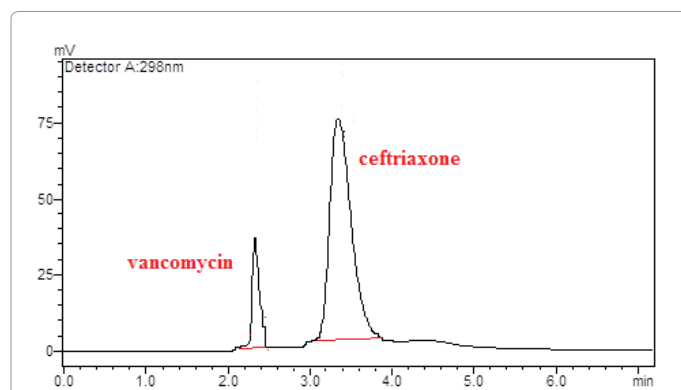


Figure 3: Chromatogram of standard ceftriaxone (60/ml) and vancomycin (30 µg/ml).

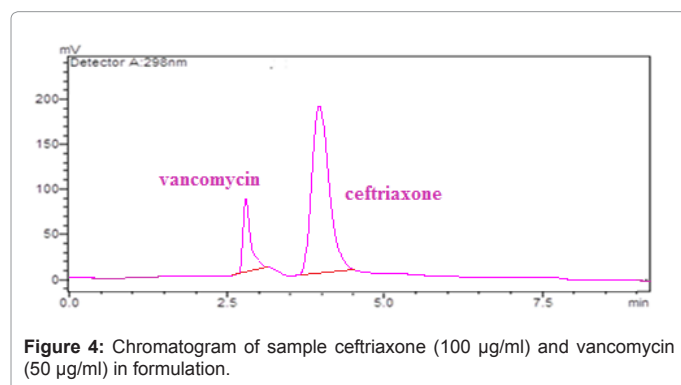


Figure 4: Chromatogram of sample ceftriaxone (100 µg/ml) and vancomycin (50 µg/ml) in formulation.

Parameters	Ceftriaxone	Vancomycin
Retention Time (Rt) min	3.7	2.7
LOD (µg/ml)	0.9	0.6
LOQ (µg/ml)	2.7	2.02
HETP	33.573	61.280
Tailing factor	1.289	1.175
Resolution	3.169	--
Theoretical plate	10467	5930
Capacity factor	13.7	24.4

Table 2: System suitability parameters of the proposed analytical method of ceftriaxone and vancomycin.

S.No	INTRA DAY				INTER DAY			
	Amount of drug taken (µg/ml)	Amount of drug found (µg/ml)	Mean	%RSD	Amount of drug taken (µg/ml)	Amount of drug found (µg/ml)	Mean	%RSD
1	30	28	29.24	1.0	30	28	28.27	1.2
2		29				28.6		
3		30				27		
4		29.6				27.6		
5		29.6				29.6		
6		28.9				28.9		

Table 3: Analytical Intraday and Interday precision of ceftriaxone.

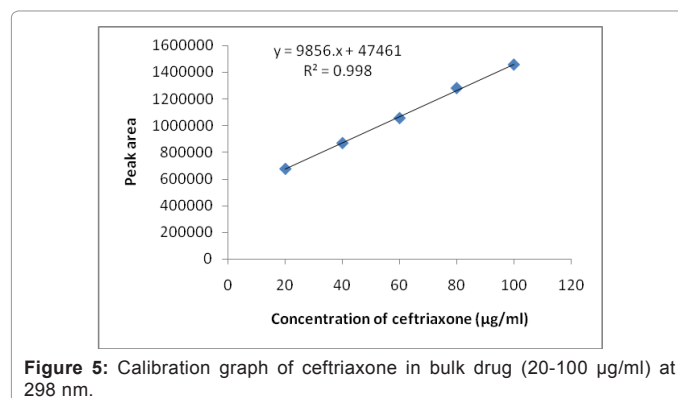


Figure 5: Calibration graph of ceftriaxone in bulk drug (20-100 µg/ml) at 298 nm.

were determined by analyzing standard working solutions. The chromatographic parameters, such as number of theoretical plates (N), resolution (Rs), capacity factor (k) and selectivity factor (α) were determined. The results are shown in Table 2, indicating the good performance of the system.

Linearity

Under the experimental conditions described above, linear calibration curves of five concentrations level for both ceftriaxone and vancomycin were obtained. The r² for ceftriaxone was 0.991 and for vancomycin sodium r²=0.9994. Linear correlations were found between peak area and ceftriaxone and vancomycin concentration (Figures 5 and 6) and are described by the regression equation. Results are specified in Table 3. The linearity range for ceftriaxone is 20-100 µg/ml and for vancomycin 10-50 µg/ml.

Precision

Analyzed a homogenous sample separately 6 times for intraday and interday using the same instrument employing the same analyst evaluated the same data for standard deviation relative standard deviation and coefficient of variance and the results are summarized in Tables 3 and 4.

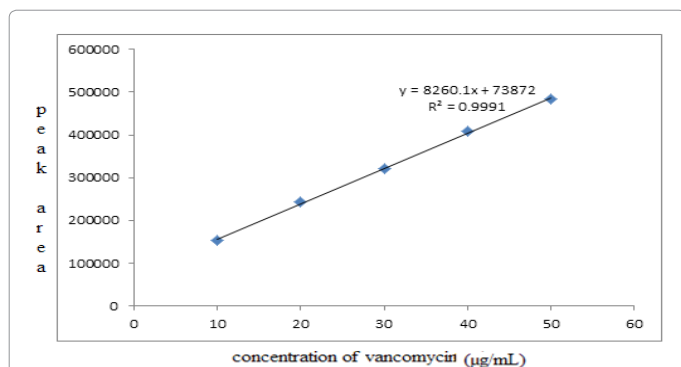


Figure 6: Calibration graph of vancomycin in bulk drug (10-50 µg/ml) at 298 nm.

S.No	INTRA DAY				INTER DAY			
	Amount of drug taken (µg/ml)	Amount of drug found (µg/ml)	Mean	%RSD	Amount of drug taken (µg/ml)	Amount of drug found (µg/ml)	Mean	%RSD
1	30	28	29.54	1.02	30	28	28.27	0.98
2		29				28		
3		30				27		
4		29.6				27.9		
5		29.6				28.7		
6		28.9				28.9		

Table 4: Analytical Intraday and Interday precision of vancomycin.

S.NO	Percent of recovery	%Recovery of ceftriaxone	%Recovery of vancomycin
1	80%	100.1	99.8
2	100%	102.1	101
3	120%	101.1	100

Table 5: Percent recovery studies of analytical method for ceftriaxone and vancomycin.

S. NO	Formulation Brand Name	Amount of ceftriaxone mg/ml		%Label Claim of ceftriaxone	%Label Claim of ceftriaxone		%Label Claim of vancomycin
		Labeled	Estimated		Labeled	Estimated	
1.	Vancopluse	1 gm	1.002 mg/ml	100.21	0.5 g	0.51 mg/ml	102.2

Table 6: Analysis of Marketed Drug.

Accuracy

To establish the accuracy of the test method, sample solutions in triplicate by spiking the test solutions with Ceftriaxone and vancomycin at 80%, 100% and 120% of the specification were prepared separately and injected into HPLC system as per the test procedure. The 'amount added,' 'amount found' and average % recovery for Ceftriaxone and vancomycin at 80%, 100% and 120% spike levels were calculated and the results are summarized in the Table 5. Assay values both the drugs are shown in the Table 6.

Limit of detection

The limit of detection is the parameter of limit tests. It is the lowest level of analytes that can be identified, but not necessarily determined in a quantitative manner, using a specific method under the required experimental conditions.

$$LOD = 3.3 S_a/b$$

Where, LOD=Limit of detection

S_a =standard deviation of the intercept

b=slope of the calibration curve

Limit of quantitation

The limit of quantitation is the least concentration of analyte in a sample that may be determined with acceptable accuracy and precision when the required procedure is applied.

$$LOQ = 10 S_a/b$$

Where, LOQ=Limit of quantification

S_a =standard deviation of the intercept

b=slope of the calibration curve

Robustness

The robustness of an analytical procedure has been defined by the ICH as a "measure of its capacity to remain unaffected by minor, but deliberate variations in method parameters. The most important aspect of robustness is to develop methods that develop methods that allow for expected variations in method parameters. Here according to ICH guidelines, robustness should be considered initial in the development phase of a method. The typical variations studied under this parameter are wavelength, flow rate, mobile phase composition, temperature, pH of the mobile phase and the results are shown in Table 7.

Stability

The Specificity indicating study of ceftriaxone and vancomycin was undergoes acid, alkali and oxidation degradation photolysis and heat condition.

Acid hydrolysis: Ceftriaxone and vancomycin solution of 100 µg/ml was treated with 1ml of acid (1N HCl) and kept heating for 1 hr. After 1 hr the solution was neutralized with 1N NaOH analyzed using HPLC.

Oxidation: Ceftriaxone and vancomycin solution of 100 µg/ml was mixed with 5 mL of 20% aqueous hydrogen peroxide solution and heating for 60 min.

Alkali hydrolysis: Ceftriaxone and vancomycin solution of 100 µg/ml was treated with 5ml of alkali (1N NaOH) and kept heating for 50min. After heating the solution was neutralized with 1N HCl.

Parameters	Ceftriaxone RSD%	Vancomycin RSD%
pH-4	1.47	1.25
pH-4.2	0.98	1.13
Flow rate 1.0 ml/min	0.9	1.26
Flow rate 1.2 ml/min	1.29	1.16

Table 7: Robustness of the method.

S.no	Stress condition	Ceftriaxone (60 µg/ml)		Vancomycin (30 µg/ml)	
		Recovered %	Degraded %	% Recovered	Degraded %
1	Acid hydrolysis (0.1N HCl)	84.66	15.4	93.32	6.68
2	Base hydrolysis (0.1 N NaOH)	76	24	90	10
3	Oxidation (20% H ₂ O ₂)	90.74	9.26	97.02	3.98
4	Thermal (700C)	89.96	10.04	87.67	12.33

Table 8: Stability studies of the ceftriaxone and vancomycin.

Photolysis: Samples were kept under UV light for different time intervals (15 mins-1 hr) and observed by HPLC.

Heat: Samples were heated at 800°C for 15 mins-60 mins and results are summarized in Table 8.

Conclusion

In the present work, a simple and accurate HPLC method for the simultaneous determination of ceftriaxone and vancomycin. The method was validated according to ICH guidelines. From the chromatogram (Figure 3) good separation ceftriaxone and vancomycin was performed at retention time of ceftriaxone (3.7) and vancomycin (2.3) were observed with a correlation coefficient (r^2) 0.9989 for ceftriaxone and vancomycin (r^2) 0.999. The limit of detection (LOD) was calculated and found to be 0.9 µg/ml ceftriaxone and 0.69 µg/ml vancomycin. Limit of quantification (LOQ) was found to be 2.7 µg/ml ceftriaxone and 2.02 µg/ml vancomycin. Intraday precision values %RSD values were found to be 1.0% for ceftriaxone and 1.025% for vancomycin. And interday precision values 1.02% and 0.988% for ceftriaxone and, vancomycin respectively. So the proposed method is more precise, accurate and robust. System suitability parameters were studied by injecting the working standard solution (20 µg/mL) is tabulated in Table 2. The optimized chromatographic for simultaneous estimation of ceftriaxone and vancomycin with good resolution can be used for evaluating in the pharmaceutical companies and research laboratories for routine and biological sample analysis.

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