



## Development of method of analysis for estimating the Vancomycin in blood plasma by RP-HPLC method: Application to *in vivo* Studies

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### Abstract

A simple, precise and reproducible reverse phase, isocratic high performance liquid chromatographic method was developed and validated for the quantitative determination of Vancomycin in blood plasma. The quantification was carried out using a HPLC system consisted of a Jasco UV- 975 intelligent UV visible detector, Jasco; PU-980 intelligent HPLC pump, Hypersil BDS C18 ; 150 mm x 4.6 mm , Clarity lite data apex 2003 software, REMI centrifuge machine (electrometer corporation); Model-R8C, with a mobile phase consisting of 10% acetonitrile in a 25 mmol/L or 50 mmol/L phosphate buffer in pH 7.0 and pH 3.2, at a flow rate of 1.0 and 1.5 ml/min ,detection wavelength were used at 229 and 270 nm. The method was validated and developed for selection of suitable Internal Standards, linearity, accuracy, robustness and solution stability. The linearity of the proposed method was investigated in the range of 5-100 µg /ml,  $R^2 = 0.995$  and  $0.997$  for reference standard vancomycin solution and in blood serum without any internal standard respectively and  $0.998$  with ondanosatrone when it was used as a I.S. The solution stability of vancomycin was studied at pH 7.0 and pH 3.2 under the same ionic strength condition at 37°C. A stability indicating HPLC method specific for vancomycin was developed and validated for use in the research. The proposed method was developed to apply for the analysis of vancomycin in bulk forms and also, the method was extended for determination of various pharmacokinetic parameters of vancomycin from *in vivo* blood plasma serum.

**Keywords:** Vancomycin, Blood plasma; Reversed-phase; HPLC, Stability

## Introduction

Vancomycin (VCM) is widely used to treat Methicillin-resistance *Staphylococcus aureus* (MRSA) infection. An efficient laboratory method is necessary to enable rapid determination of its concentration in blood [1]. It is most commonly used in the treatment of virulent gastrointestinal or systemic infection, such as those elicited by *Staphylococcus* and enterococcal organism [2, 3]. Glycopeptide antibiotics such as vancomycin, ramoplanin and teicoplanin are life saving drugs in certain clinical situations where first line antibiotics (e.g. penicillins, cephalosporins) result in treatment failure [4, 5].

High-performance liquid chromatography (HPLC) is the most reliable approach to the assay and at the same time can be used as a standard for comparison with other methods although it requires complicated pretreatments for sample preparation. A number of HPLC VCM assays have been developed using solid –phase extraction [6, 7], protein precipitation [8] followed by extraction into an organic solvent [9-11] for sample preparation. However, to develop a more rapid and sensitive method suitable for routine use, it is necessary to eliminate the sample preparation step. We had developed a vancomycin measurement method using HPLC that combines column switching and part-time recycling of the mobile phase employing direct injection of micro quantities of serum [12].

We confirmed the utility of this method for clinically laboratories. However, simplification of the measurement system was necessary to improve maintenance, management, and operation. We therefore designed a vancomycin measurement method by isocratic HPLC whereby serum is directly injected into the separation column using a semi permeable surface (SPS) packing material [13].

The primary route of vancomycin degradation occurs by the deamidation of the asparagines residue proceeding through two mechanisms: general acid or base catalyzed hydrolysis or by the formation of an imide intermediate to yield degradation products CDO-1 or CDP-1m [5, 14]. The controlled study of the solution stability of vancomycin at pH 7.0 and pH 3.2 at 37°C has not been reported in the literature. The study of vancomycin stability in these media could demonstrate the relevant importance of hydrolytic degradation mechanism. The stability study of vancomycin at pH 7.0 and pH 3.2 was carried out to ensure the drug remain stable during the period of analysis by HPLC. The proposed method was developed to apply for the analysis of vancomycin in bulk forms and also, the method was extended for determination of various pharmacokinetic parameters of vancomycin from *in vivo* blood plasma serum.

## Materials and Methods

### Chemicals and reagents

Vancomycin reference standard, and internal standards (IS); imipramine, nebivolol, ondansatrone, aceclofenac and ibuprofen were procured from CDL (Central Drug Laboratory), Kolkata, West Bengal, India. All solvents used were of HPLC grade. Acetonitrile, methanol, potassium di-hydrogen phosphate, di-sodium hydrogen phosphate anhydrous and sodium hydroxide were obtained from E. Merck, Mumbai, India. HPLC grade water was obtained by passage through a Milli-Q system (Millipore, Milford, MA, USA).

**Instrumentation**

A Jasco HPLC system was used for development of analytical method. The system equipped with a Jasco UV- 975 intelligent UV-Visible detector, Jasco; PU-980 intelligent HPLC pump, Hypersil BDS C<sub>18</sub> (150 x 4.6 mm), 4 μm was used as a stationary phase.

Clarity lite (clarity- chromatography SW) data apex, 2003 software and REMI company (electrometer corporation); Model-R8C Centrifuge machine, 0.22 μm membrane filter were used.

**Chromatographic conditions**

The chromatographic column used was a 150 mm × 4.6 mm, Hypersil BDS C18, with 4 μm particles. The flow rate of the mobile phase was maintained at 1.5 ml /min and the column temperature 25°C. Detection was carried out at 229 nm of maximum wavelength, the injection volume was 5-100 μL and the run time was monitored upto 10 min.

**Mobile phase preparation and preparation of Standard samples**

The preparation of standard curve was modified as followed as reported from some earlier methods [1, 5]. Mobile phase was prepared and which was composed of 10% acetonitrile in a 25 mmol/L phosphate buffer in pH 7.0 (1.701 gm of KH<sub>2</sub>PO<sub>4</sub> mixed with 500 ml HPLC water and pH adjusted to 7.0 ). The solution was filtered in membrane filter pore size 0.22 μm and sonicated properly. About 100 ml of phosphate buffer (pH 7.0) was separated for initial washing of HPLC machine after washing with methanol and rest 400 ml was further diluted with 5% of acetonitrile (10 %) and after mixing and sonicating properly, it was used for running chromatogram.

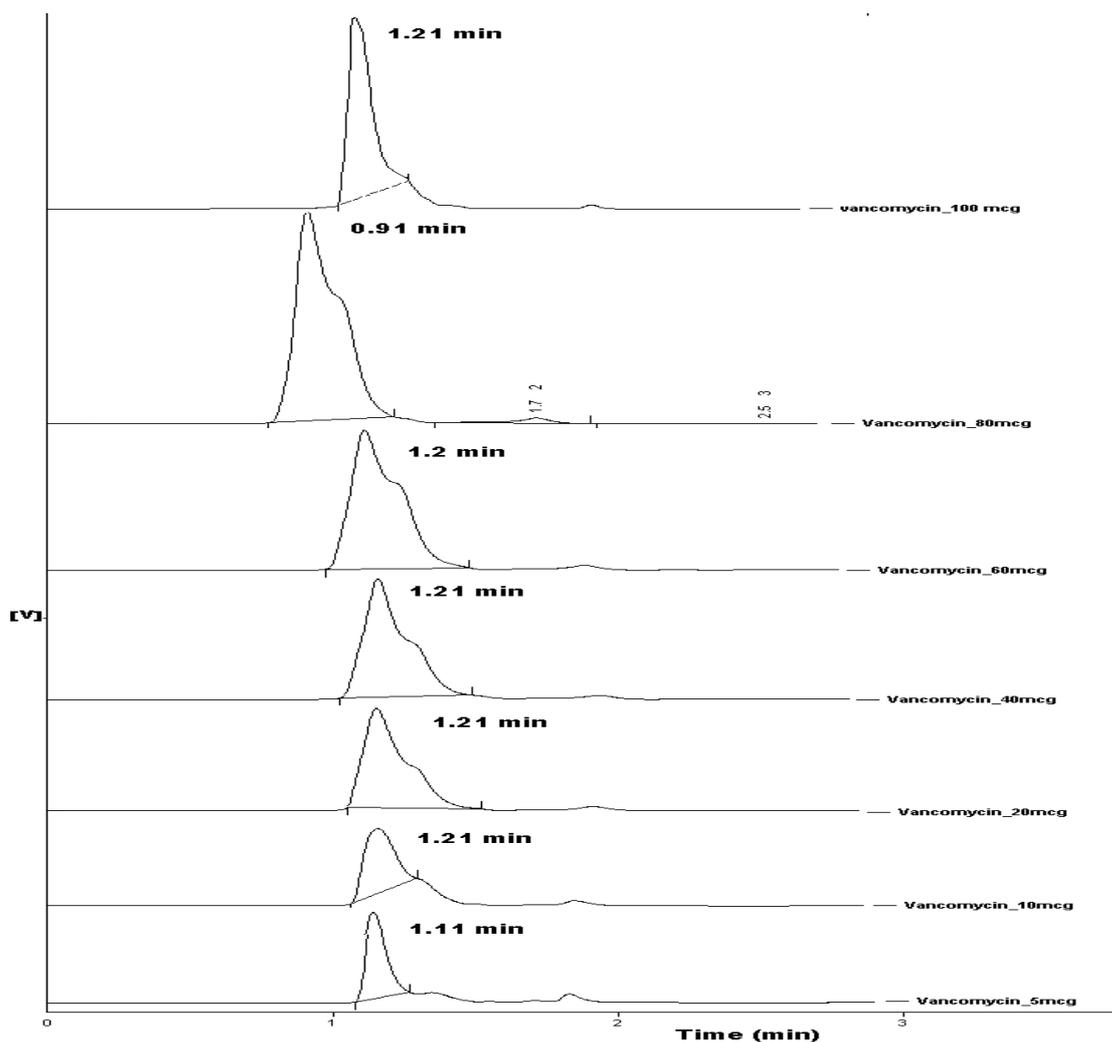
Standard samples were prepared in 5, 10, 20, 40, 60, 80 & 100 mcg /ml of vancomycin reference standard in methanol and sonicated well after filtration. Each sample was runned one by one by injecting 5-100 μL into the injecting port; after washing the HPLC machine, initially with methanol followed by buffer solution, in a flow rate of 1.5 ml/min, detection was carried out at 229 nm, and washing time with mobile phase itself was given to 10 min.

**Data analysis**

Data from method validation runs were analyzed liner and log linear regression models were used to estimate the values of method validation and stability parameters, respectively. One ay analysis of variance and analysis of covariance subroutines were used to determine the statistical significance of the estimated parameters. A critical probability level of  $\alpha=0.5$  was used to determine statistical significance.

**Results and Discussion**

Each and every standard solution of vancomycin (fig. I) in the range of 5-100 μg/ml of samples were showing retention time in the range of 0.91 min-1.21min i.e., peak time. The runned samples were plotted and calibrated with software “Clarity lite” and allowed to store as a recorded to make a standard curve and to see the linearity of the areas vs concentrations, which is checked by linear regression coefficient value ( $R^2$ ) ≥ 0.98. These helps later to identify the vancomycin concentration in patient’s serum sample in respect to standard plotted graph.

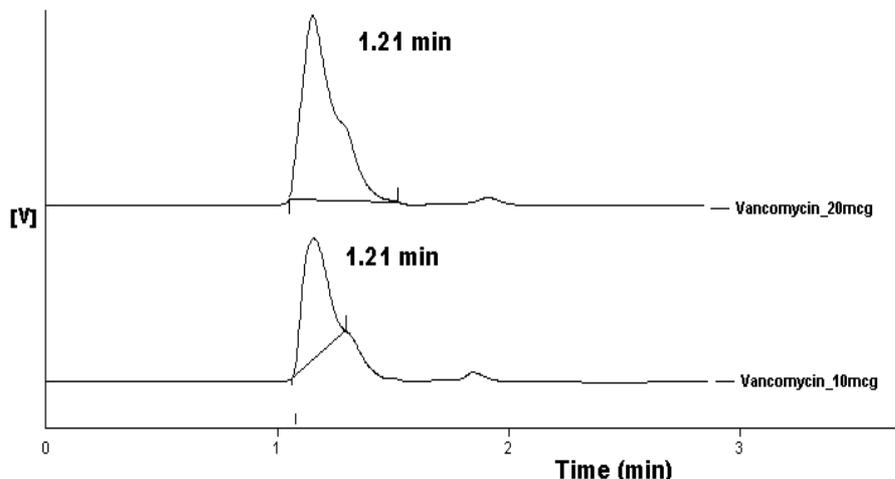


**Fig. I. Chromatograms of Vancomycin standard solution of 5, 10, 20,40,60,80, and 100 mcg/ml**

### Method development

To develop a precise, accurate and suitable HPLC method for quantitative determination of vancomycin from any formulation and presence of vancomycin in patient blood serum, different mobile phase and some chromatographic conditions were altered.

To developed a suitable method of analysis by HPLC the author tried to increase the retention time by changing the flow rate 1ml/min instead of flow rate 1.5 ml/min and buffer concentration changed to 50 mmol/L from 25 mmol/L at the same pH( 7.0), but which did not change the retention time, its was still on 1.21min (fig II.), but the retention time should be extent as we knows if the serum has more than one drug which can overlaps and interfere with the peak as well as AUC. On looking to the limitation of chemicals the author tried to plot graph by running the chromatogram with 10 and 20 mcg/ml sample as shown in fig II.



**Fig. II. Chromatograms of vancomycin standard solution of 10 and 20mcg/ml**

No significance change noticed in increase the retention time. Author's main objective to increase the retention time to get the marked vancomycin peaks in plasma samples which contains many drugs, to avoiding the overlapping interference of peaks. So, we followed the earlier reported method [12, 15] and altered some variables like wavelength, flow rate, run time, temperature, pH of phosphate buffer and mobile phase. Firstly prepared acidic buffer solution (pH 3.2) (50 mmol/L) instead of 25 mmol/L Phosphate buffer (pH 7.0).

Mobile phase was used composed of acetonitrile (10%): HPLC water in ratio 5:95 and measured at 270 nm instead of 229 nm, in a flow rate 1 ml/min at 25°C and run time was monitored up to 5-10 min.

As shown in fig-III, by altering some chromatographic variables author able to increased the retention time from 1.21 min to 2.57 min and as well as got a calibration curve with a higher regression coefficient.

### Method validation

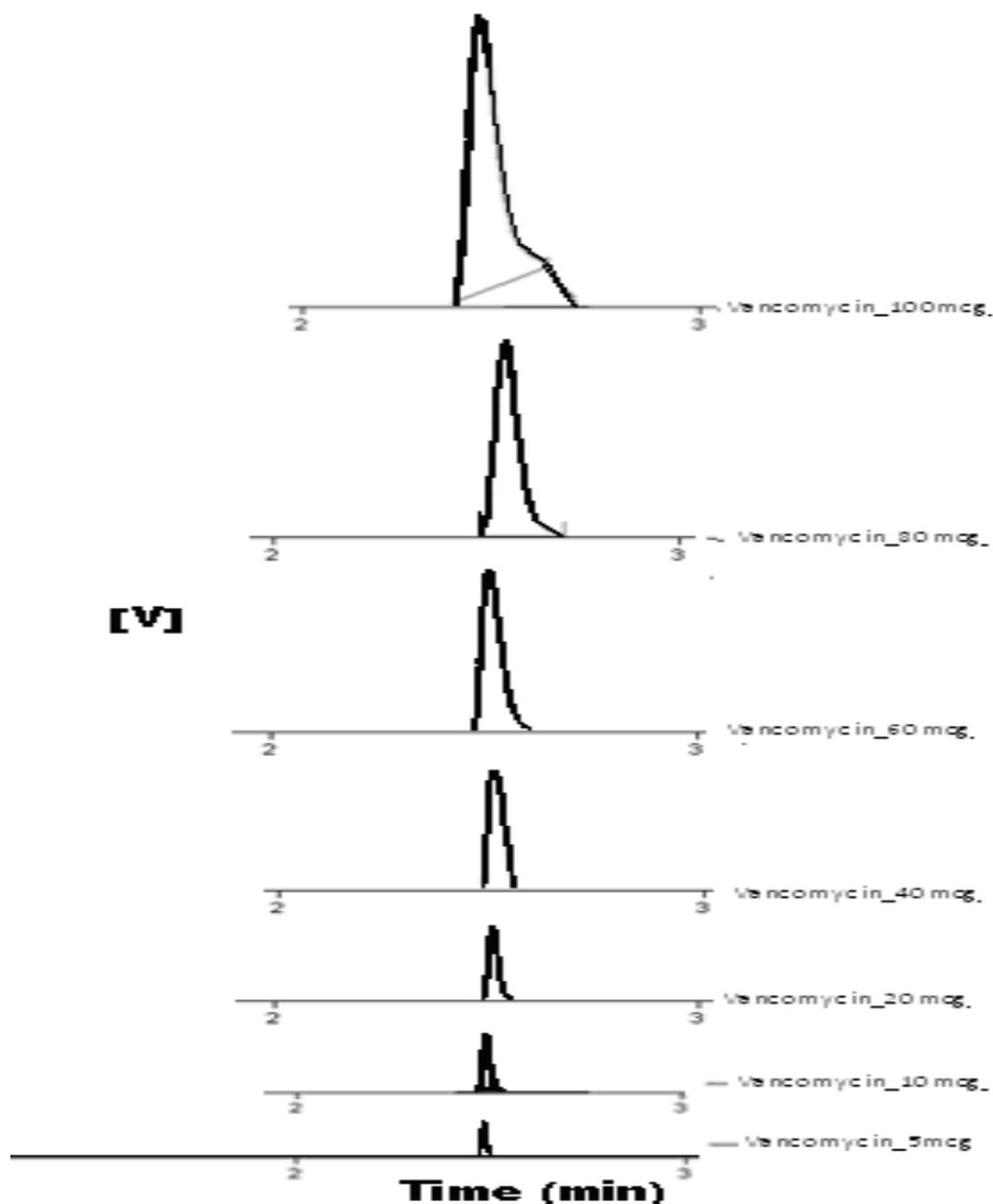
#### Linearity

Linearity was studied by preparing solutions at different concentration levels when the concentration of vancomycin and its respective peak areas were subjected to regression analysis by least squares method, a good linear relationship ( $r^2=0.995$ ) and ( $r^2=0.997$ ) were observed between the in the concentration range of 5-100  $\mu\text{g/ml}$ , for respective reference standard solution of drug and vancomycin with blank blood plasma respectively, with a regression equation  $Y=0.5091X+0.0624$  and  $Y=0.0509X+ 0.4376$ , where Y is the peak area (AUC) in mV.s and X is the concentration of vancomycin in  $\mu\text{g/ml}$ .

#### Selection of suitable Internal Standards

Internal standards (IS) are very much essentials for the bioassays. Here, in this HPLC assay [16] author was used four Internal Standards (IS) – imipramine, nebivolol, ondanosatron, aceclofenac, and ibuprofen. Imipramine and nebivolol having nearly same retention time (RT) 2.56 min and

2.54 min respectively as Vancomycin has in the serum plasma, so its not wise to use as IS as it was not showed the separate peaks. Aceclofenac also showing RT at 2.49 min, while the Ibuprofen showing RT at 2.36 min but ondanosatrone showing RT at 2.83 min. If the RT of IS is much more similar to that of vancomycin may not suitable as an IS, in order to avoid interference with serum plasma components during the analysis of vancomycin. So, according to the need of selection of IS, ondanosatrone was selected and run was done in HPLC with vancomycin in serum as parent drug in different concentration ranges.



**Fig. III.** Chromatogram of vancomycin standard solutions (5-100 mcg/ml) by altering some variables as mentioning in preparation of standard solution.

500 microL of blood samples were collected from 11 nos. of pretreated patients with vancomycin and which was initially stored at -20 °C before used. 500 microL of di-methylsulfoxide [DMSO] (which proved to be purely soluble for drugs and Internal Standard) and 500 microL of blood samples was shaken properly and centrifuged at 5000 rpm to precipitate and remove unwanted proteinous residues. Supernatant liquid was collected which is spiked serum containing vancomycin, other antibiotics and concentration of IS was maintained to 60 mcg/ml to it.

From the supernatant liquid 20 microL of the spiked serum was then collected individually from each 11 patient's and samples were run in HPLC, which is having known concentration 60 mcg/ml of IS and unknown drug concentration.

These spiked samples of 11 patients run with the help of following chromatographic methods and its specificity of chromatographic condition for analysis of blood serum of vancomycin with known concentration (60 mcg/ml) of IS were mentioned as followed: It was very much needed to increase the retention time of the sample, as it was ruined with the spiked plasma and the plasma itself gave a peak which shows within 2 or 3 RT, also plasma contains other blood contents. So it may interfere with the expected peaks of vancomycin and may give poor result and so its need to alter or increase the RT of Vancomycin. When it was observed that by altering the flow rate i.e., decreasing flow rate, the RT increases very less. Then few alterations were done, which was as followed and used for development of method in standardization of vancomycin to get an efficient result: 1] Altering the concentration of Phosphate buffer from 25 mmol/L to 50 mmol/L. 2] Flow rate altered from 1.5 ml/min to 1ml/min. (If it was reduced to 0.8 ml/min then it did not show firm peaks) 3]. The wavelength was changed from 229 nm to 270 nm gradually to adjust the baseline and it was selected on the basis of earlier reported method [15]. 4] pH of the buffer was made by changing and adjusting up to 3.2 gradually.

Finally developed method showed alters of RT from 1.21min to 2.57 (fig III and table III). From the above chromatograms the following data were evaluated: From the corresponding known value of AUC and concentration (60µg/ml) of Internal Standard and known values of AUC of Vancomycin ( table IV), author able to evaluate the values of Vancomycin concentration of each patient's blood samples.

#### **Method accuracy**

To ensure the reliability and accuracy of the method, recovery studies were carried out in triplicate at three concentration levels (50%, 100% and 150%) of test concentration. The recovery of vancomycin was found to be in the range of 99.3-101.1 %.

#### **Stability of standard solution**

The solution (pH 7.0) stability of vancomycin was carried out by leaving the test solutions in a tightly capped volumetric flask at 37 °C. The same sample solutions were assayed for a predetermined time interval up to the study period against freshly prepared solutions. The stability of vancomycin solution at pH 7.0 and pH 3.2 was very good and the first order rate constant and  $t_{50}$  was  $1.31 \times 10^{-3}$  ( $h^{-1}$ ), 21.9 days for pH 7.0 and  $2.02 \times 10^{-3}$  ( $h^{-1}$ ), 14.4 days for pH 3.2.

**Method robustness**

This was done by small deliberate changing in flow rate, pH of mobile phase, mobile phase concentration and wavelength. Results were shown in fig III and table I, II and III. Results showed that the contents of the drug were not adversely affected by these changes which indicating that the method was robust.

**Table- I: Retention, Hight and Areas of chromatograms of simple vancomycin solution.**

Conc. (mcg)	Retention Time(min)	Height of Peaks [mV]	Area of Peaks [mV.s]
5	2.57	0.012	0.12243
10	2.57	0.021	0.24587
20	2.57	0.031	0.48974
40	2.57	0.049	0.85443
60	2.57	0.051	1.28165
80	2.57	0.10	1.7088
100	2.57	0.148	2.136

**Table II: Retention, Hight and Areas of chromatograms in Serum without use of any I.S.**

Conc. (mcg)	Retention Time(min)	Height of Peaks [mV]	Area of Peaks [mV.s]
5	2.57	0.014	0.0921
10	2.57	0.026	0.2133
20	2.57	0.031	0.4199
40	2.57	0.048	0.8501
60	2.57	0.050	1.275
80	2.57	0.11	1.7002
100	2.57	0.14	2.127

**Table III: Retention, Hight and Areas of chromatograms in Serum in presence of ondanosatron as IS.**

Conc. In µg	Retention Time Vancomycin	Retention Time of I.S.	Area of Peaks AUC [mV.s] for I.S. of 100 µg	Area of Peaks AUC [mV.s] for Vancomycin
5µgm	2.57 min	2.83 min	2.275	0.113
10µgm	2.57 min	2.83 min	2.275	0.208
20µgm	2.57 min	2.83 min	2.275	0.460
40µgm	2.57 min	2.83 min	2.275	0.905
60µgm	2.57 min	2.83 min	2.275	1.52
80µgm	2.57 min	2.83 min	2.275	1.827
100µgm	2.57 min	2.83 min	2.275	2.269

**Table IV: Analysis of vancomycin after collecting from various patients' blood containing vancomycin with help of developed standard curve and chromatographic condition with known concentration of I.S.**

Patient's Samples PEAK	PS <sub>1A</sub>	PS <sub>2A</sub>	PS <sub>3A</sub>	PS <sub>4A</sub>	PS <sub>5A</sub>	PS <sub>6A</sub>	PS <sub>7A</sub>	PS <sub>8A</sub>	PS <sub>9A</sub>	PS <sub>10A</sub>	PS <sub>11A</sub>
AUC of known I.S. conc. 60µg (in mV.s)	1.284	1.284	1.284	1.284	1.284	1.284	1.284	1.284	1.284	1.284	1.284
AUC of unknown conc. Vancomycin (in mV.s)	0.963	0.988	0.975	0.941	1.027	0.800	0.981	0.984	0.981	0.982	0.990
Concentration of Vancomycin evaluated (in µg/ml)	45.0	46.2	45.6	44.0	48.0	37.40	45.87	46.0	45.85	45.90	46.27
Patient's Samples TOUGH	PS <sub>1B</sub>	PS <sub>2B</sub>	PS <sub>3B</sub>	PS <sub>4B</sub>	PS <sub>5B</sub>	PS <sub>6B</sub>	PS <sub>7B</sub>	PS <sub>8B</sub>	PS <sub>9B</sub>	PS <sub>10B</sub>	PS <sub>11B</sub>
AUC of known I.S. conc. 60µg (in mV.s)	1.271	1.273	1.271	1.272	1.272	1.271	1.278	1.279	1.276	1.270	1.279
AUC of unknown conc. Vancomycin (in mV.s)	0.338	0.297	0.317	0.330	0.281	0.360	0.383	0.366	0.299	0.258	0.234
Concentration of Vancomycin evaluated (in µg/ml)	16.0	14.0	15.0	15.6	13.3	17.0	18.0	17.2	14.1	12.2	11.0

## Conclusion

So, from these above given data it can be able to evaluate the *in vitro* parameters, drug contents from any vancomycin containing formulations as well as the Pharmacokinetic Parameters likes – Elimination rate constant ( $K_{el}$ ) ; Biological half life ( $t_{1/2}$ ) ; Volume of distribution ( $V_d$ ); Maintenance dose (MD) and Vancomycin Clearance. The proposed method can be successfully applied for the analysis of vancomycin in bulk and pharmaceutical dosage forms. Also, the method was extended for determination of vancomycin *in vivo* blood plasma serum.

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