Bio-Analytical Method Development and Validation of Antihypertensive Drug by RP HPLC Method

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Abstract: The present study outlines the development and validation of a precise, accurate, and sensitive reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of Vericiguat, a soluble guanylate cyclase (sGC) stimulator indicated for the treatment of heart failure with reduced ejection fraction. Chromatographic separation was achieved using a Hypersil,C18(250mm x 4.6ID,particle size :5um) column, with a mobile phase composed of MeOH : H_2O:TFA, in proportion of 75:25:0.1 respectively, at a flow rate of 1.0 ml/min. Detection was carried out at a wavelength of 257 nm. The method was validated in accordance with ICH and USFDA bioanalytical guidelines, evaluating key parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), recovery, and stability. Vericiguat exhibited a retention time of approximately 3.746 minutes. Linearity was established over the concentration range of 10-30ug/ml, yielding a correlation coefficient (R^2) of 0.99997. The method demonstrated acceptable intra- and inter-day precision, with relative standard deviation (RSD) values within permissible limits. Recovery studies showed consistent and reproducible results, confirming the efficiency of the extraction process from biological matrices. This validated RP-HPLC method proves to be reliable and suitable for routine bioanalytical applications, including the quantitative estimation of Vericiguat in human plasma and potential use in pharmacokinetic and therapeutic drug monitoring studies.

Index terms : High Performance Liquid Chromatography (HPLC), Vericiguat, Hypertension, Validation, System suitability.

INTRODUCTION:

The development and validation of bioanalytical methods are essential steps in the assessment of drug concentrations within biological samples such as blood, plasma, or urine. Reverse Phase High-Performance Liquid Chromatography (RP-HPLC) is one of the most widely used techniques for this purpose, owing to its accuracy, reproducibility, and ability to handle complex biological matrices. This method separates analytes based on their interaction with a non-polar stationary phase and a polar mobile phase, making it well-suited for analyzing a wide range of drug compounds. During method development, various parameters such as mobile phase composition, flow rate, and detection wavelength must be carefully optimized. Once the method is established, it undergoes a validation process to confirm its reliability, precision, and compliance with regulatory standards, ensuring it is fit for use in pharmacokinetic, clinical, and stability studies[1].

Hypertension, commonly known as high blood pressure, is a chronic medical condition where the force of the blood against the walls of the arteries remains consistently elevated. It is often referred to as a "silent killer" because it typically develops without noticeable symptoms, yet it can lead to serious health complications such as heart disease, stroke, kidney damage, and other cardiovascular issues if left unmanaged. Hypertension can be influenced by a variety of factors, including genetics, age, lifestyle habits, and underlying medical conditions. With the increasing prevalence of sedentary lifestyles and unhealthy diets, hypertension has become a significant global health concern. Early detection, lifestyle modifications, and appropriate medical treatment are essential in managing this condition and reducing the risk of long-term complications[2].

Vericiguat is a novel oral medication developed to treat certain forms of heart failure, particularly heart failure with reduced ejection fraction (HFrEF). It functions by enhancing the nitric oxide (NO)-soluble guanylate cyclase (sGC) signaling pathway, which plays a vital role in cardiovascular regulation. By stimulating sGC, vericiguat helps relax blood vessels and improve blood flow, thereby reducing the workload on the heart. Approved by regulatory authorities such as the FDA, vericiguat offers a new therapeutic option for patients with worsening heart failure symptoms despite standard treatment[3].

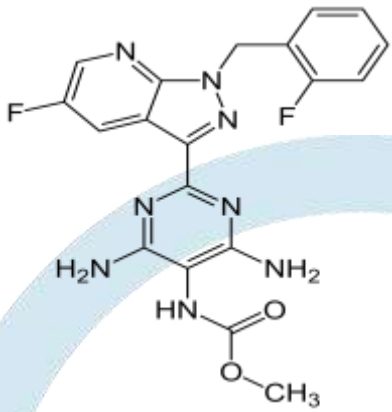

Fig No. 1 : Chemical structure of vericiguat

MATERIALS AND METHODS:

Chemicals : Vericiguat obtained from Dr.Reddy's laboratories Ltd. 8-2-337, Road no.3,banjara hills, Hyderabad, Telangana 500034, india. Dimethyl sulphoxide (DMSO) was purchased from Meru chem pvt. Ltd., Trifluroacetic acid (HPLC Grade) was purchased from Finar Pvt.Ltd., Water (HPLC Grade) was purchased from Qualigens Pvt. Ltd. , Methyl Alcohol was purchased from loba chemicals Pvt. Ltd. Mumbai.

Instruments : For the completion of present research study the given instruments were used for various purpose. UV- visible spectrophotometer – Shimadzu-1800 Double beam with UV probe 2.33. HPLC system – Younglin HPLC system, model no. 1100, [mfg- Agilent technologies (USA)] was used for method development and validation, Pump- SP930 D, Detector- UV detector (730), Software- EZ-chrome Elite, Column- Hypersil C18 (250 mm x 4.6mm,5um).

Preparation of Vericiguat standard stock solution : Accurately weighed 20mg of vericiguat standard was transferred into 100ml glass volumetric flask. The contents were dissolved and diluted to volume with the appropriate diluent to prepare the stock solution. the freshly prepared solutions was then shaken well, sonicated for 5 minutes,and subsequently filtered through a 0.22 um syringe filter prior to analysis[4].

Extraction Procedure (Protein Precipitation) : This method is commonly employed for the extraction of analytes from biological fluids. In this approach, precipitating agents such as acetonitrile, buffer, or acetic acid are added to blank plasma to precipitate the plasma proteins. The resulting precipitate is then removed by filtration or centrifugation. The supernatant obtained is directly injected into the HPLC column without any additional treatment, and chromatograms are subsequently recorded. An aliquot of 0.2 mL of plasma was transferred into a glass tube, followed by the addition of 0.2 mL of the standard solution and 1.6 mL of diluent. The mixture was then centrifuged at 5000 rpm for 15 minutes to facilitate the extraction of the drug from plasma. The percentage recovery of the drug was calculated for each of the precipitating agents used in the study. Chromatograms of plasma samples extracted using acetonitrile and buffer were recorded under fixed chromatographic conditions. Additionally, a chromatogram of blank plasma (without the drug) was also obtained for comparison. Based on the percentage recovery results, acetonitrile was selected for further studies due to its higher recovery efficiency[5].

METHOD VALIDATION :

The method performance was evaluated for accuracy, precision, linearity range, robustness, limit of quantification(LOQ), limit of detection (LOD), system suitability studies

Accuracy : In this study, selected concentrations of plasma samples were injected in triplicate, and the mean peak area for each concentration was calculated. The deviation between the observed values and the true concentrations was then determined to assess the accuracy of the method[6].

Precision : Intraday and interday precision studies were carried out to evaluate the reproducibility of the developed method. For intraday precision, plasma samples containing Vericiguat at different concentrations were analyzed multiple times within the same day, and chromatograms were recorded. For interday precision, the same procedure was repeated over a two-week period to assess variability across different days. For this precision study, samples were prepared at three quality control levels: low (LQC – 25 µg/mL), medium (MQC – 50 µg/mL), and high (HQC – 75 µg/mL)[7].

Linearity and Range : Linearity and range were assessed using a calibration curve constructed from calibration standards prepared by spiking plasma with Vericiguat at concentrations ranging from 25 to 75 µg/mL. The calibration graph was plotted with the concentration of spiked plasma samples on the X-axis and the corresponding peak area on the Y-axis. Linearity was evaluated over the range of 50% to 150% of the proposed concentration, ensuring the method's reliability across the expected analytical range[8].

Robustness : Robustness of the method was evaluated by making slight deliberate changes to the chromatographic conditions. Under these modified conditions, standard solutions were injected, and the resulting chromatograms were assessed to determine whether the method remained reliable and consistent.

- ± 5 % difference in the ratio of mobile phase.
- ± 2 difference in wavelength.
- ±. 0.5 % difference in flow rate of the mobile phase.

Under the altered chromatographic conditions, key parameters such as separation factor, retention time, and peak symmetry were evaluated to assess the robustness of the method[9].

Limit of Quantification (LOQ) : The limit of quantification (LOQ) was determined as the lowest concentration of Vericiguat on the standard calibration curve that could be reliably quantified, with a bias of less than 20% and a signal-to-noise ratio (S/N) of at least 10[10].

$$LOQ = \frac{10 \text{ X Standard deviation of the response of blank}}{\text{Slope}}$$

Limit of Detection (LOD) : The limit of detection (LOD) is defined as the lowest concentration of the analyte in a sample that can be detected but not necessarily quantified under the given experimental conditions. It represents the minimum concentration that can be distinguished from background noise with a defined level of confidence[11].

$$LOD = \frac{3.3 \text{ X Standard deviation of the response of blank}}{\text{Slope}}$$

System Suitability Studies : System suitability studies were conducted as per USP guidelines to ensure the performance and reliability of the chromatographic system. Key parameters such as column efficiency, resolution, and capacity factor were calculated through repeated injections of standard solutions. These evaluations confirmed that the system met the required analytical criteria for reliable analysis[12].

RESULTS :

Optimization of chromatographic conditions : After conducting several trials with different mobile phases, methanol was selected as the organic phase due to its favorable elution properties for the drug from the stationary phase. For the aqueous phase, a 0.5% acetonitrile solution was chosen, considering the chemical nature of the selected analytes. The ratio of organic to aqueous phase was systematically adjusted to achieve optimal separation and resolution of the compounds during HPLC analysis.The optimized chromatographic conditions and chromatogram of analyte and system suitability parameters are listed in table 1 and fig. 2.

***Table No.1*:** Optimized chromatographic conditions and system suitability parameters of selected methods

Mobile phase	MeOH:H2O:TFA, 75:25:0.1
Column	Hypersil, C18 (250mm x 4.6ID, Particle size: 5 um)
Flow rate	1.0 ml ml/min
Injection volume	20 µL.
Temperature	Ambient
Wavelength	257 nm
Run time	7 minutes
Elution mode	Isocratic
Diluent	Mobile phase
Std conc.	20 ppm (20 mg /100 ml)→ 2 ml/20 ml
Remark	TP & TF (Asymmetry) observed within acceptance criteria, peak shape is good, here method is developed and we need to go validation of this method before use,

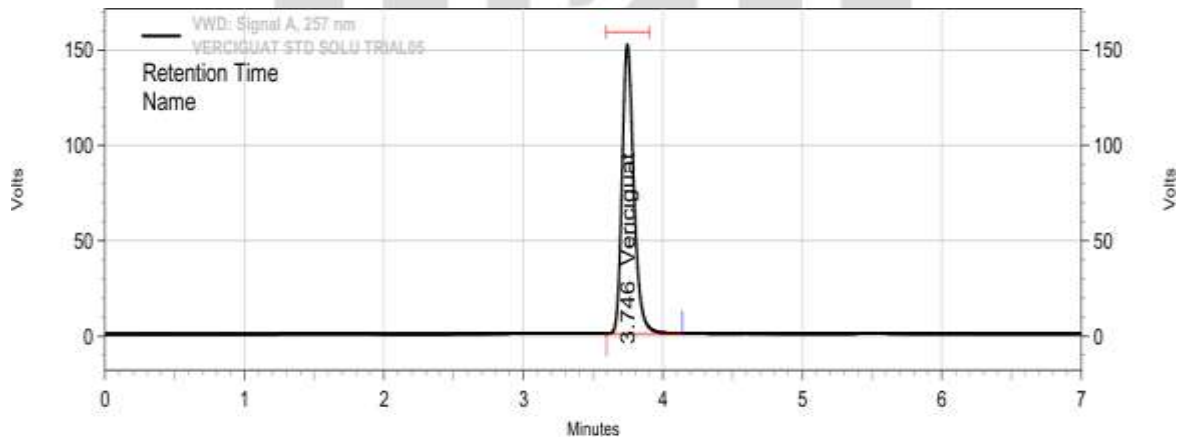

Fig 2 : Chromatogram of spiked human plasma with vericiguat standard

METHOD VALIDATION

Accuracy

Table No.2 : Accuracy data of vericiguat by HPLC method

	Mean % recovery	SD	%RSD (NMT 2)
Accuracy at 80 %	101.41	0.3160	**0.31**
Accuracy at 100 %	98.47	0.4846	**0.49**
Accuracy at 120 %	101.46	0.2023	**0.20**

% Mean recovery observed within acceptance criteria, %RSD of recovery observed within a acceptance criteria, hence Accuracy is justified.

Precision

Table No. 3 **:** Intra- day precision data of vericiguat

Name	**Preparations**	**% Assay**
Set-1	prep-1	99.76
	prep-2	100.44
Set-2	prep-1	99.89
	prep-2	99.31
Mean		99.85
SD		0.4653
% RSD (NMT 2)		**0.47**

Overall % RSD for results of set-1 and set-2 performed in single day observed within acceptance criteria, so method is precise in terms of repeated analysis in single day, hence intraday precision is justified.

Table No. 4 : Interday precision data of Vericiguat

Name	**Preparations**	**% Assay**
Day-1	prep-1	99.76
	prep-2	100.44
Day-2	prep-1	98.48
	prep-2	99.18
Mean		99.47
SD		0.8345
% RSD (NMT 2)		**0.84**

Linearity and Range :

***Table No. 5* :** Calliberation standards peak area

Con. (ppm or ug/ml)	**Area**
10.00	7385209
15.00	11097334
20.00	14863694
25.00	18697835
30.00	22385108
Correlation coefficient	NLT 0.995
Intercept	154284
Slope	752006

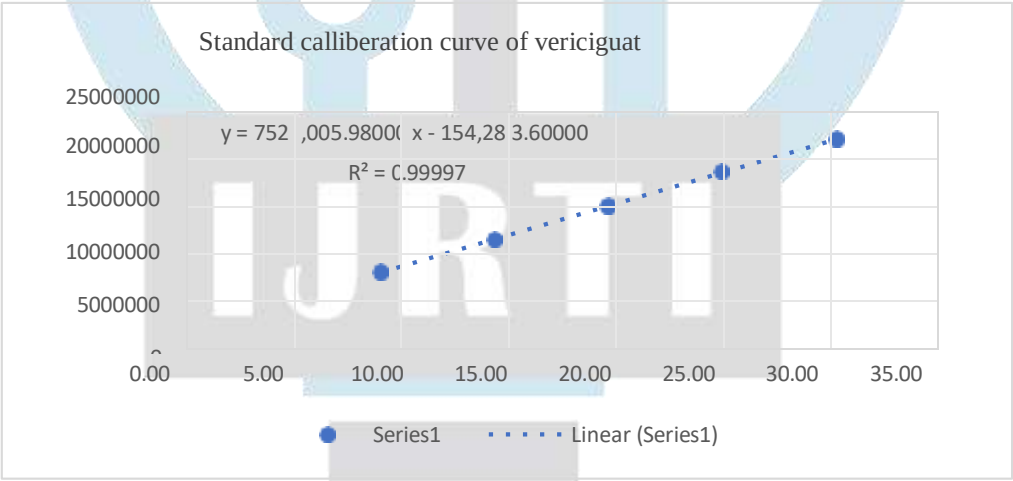

Fig 3 : Standard calliberation curve of vericiguat

Corellation cofficient observed within acceptance criteria, hence method is linear and linearity is justified.

Robustness :

Table No. 6 : Robustness change in method parameter

Name	**Preparations**	**%Assay**
Robustness change in method parameters		
Original method parameters	Test prep-1	99.76
Original method parameters	Test prep-2	100.44
MeOH:H2O:TFA, 70:30:0.1	Test prep	99.01
MeOH:H2O:TFA, 80:20:0.1	Test prep	99.84
Wavelength 255 nm	Test prep	98.05
Wavelength 259 nm	Test prep	98.38
Mean		99.25
SD		0.9251
%RSD (NMT 2)		**0.93**

Overall % RSD of results with change in mobile phase composition & change in wavelength observed within acceptance criteria. Method is robust in terms of slight change in internal method parameter , hence robustness is justified.

Limit of Quantification (LOQ) and Limit of Detection (LOD) :

Table No. 7 : LOQ and LOD data of vericiguat

Con. (ppm or ug/ml)	**Area**
10.00	7385209
15.00	11097334
20.00	14863694
25.00	18697835
30.00	22385108
STEYX	40048
SLOPE	752006
LOD (ug/ml)	0.18
LOQ (ug/ml)	0.53

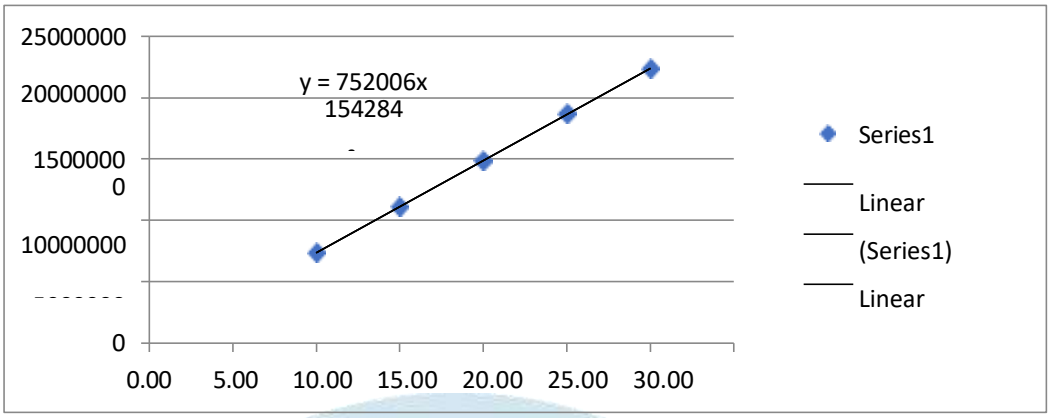

Fig No. 4 : LOQ and LOD graph of vericiguat

System Suitability :

Table No. 8 : System suitability

Name	**Area**	**RT(min)**	**TP (NLT 2000)**	**TF (NMT 2)**
Standard _Inj_01	14776178	3.737	9888	1.37
Standard_Inj_02	14844105	3.737	9871	1.39
Standard_Inj_03	14823811	3.737	9925	1.33
Standard_Inj_04	14840929	3.732	9949	1.33
Standard_Inj_05	14838068	3.732	9957	1.36
Mean	**14824618**	**3.735**		
SD	28168.1524	0.0027		
%RSD (NMT 2)	**0.19**	0.07		

Theorotical plates and Tailing factor observed within acceptance criteria, also % RSD of replicate injections for area and retention time observed within acceptance criteria, here system is suitable for analysis of Vericiguat. Hence System suitability is justified.

CONCLUSION :

In this study, a robust, precise, and accurate reverse-phase high-performance liquid chromatography (RP-HPLC) method was successfully developed and validated for the bioanalytical quantification of vericiguat. The method demonstrated excellent linearity, specificity, sensitivity, and reproducibility in accordance with ICH and FDA guidelines. Key validation parameters including accuracy, precision, robustness, system suitability study, limit of detection (LOD), and limit of quantitation (LOQ) were well within acceptable limits, confirming the reliability of the method for routine bioanalytical applications. This validated method can be effectively applied for pharmacokinetic studies, therapeutic drug monitoring, and quality control of vericiguat in biological matrices.

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