

QbD Based Analytical Optimization by UV Spectroscopy Assisted HPLC and HPTLC Methods for Quantification of Voclosporin in Pharmaceutical Formulation

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ABSTRACT

Background: The innovative analytical quantification of lupus medication Voclosporin was developed by HPLC, and HPTLC was abetted by the Ultra Violet spectroscopic method using the QbD approach. **Materials and Methods:** The Cary 5000 double-beam spectrophotometer was used to measure the UV absorbance. Chromatographic separation was performed in the Agilent C₁₈ column with mobile phase consisting of different concentrations of Acetonitrile and phosphate buffer. Variables of the methods were optimized by response surface methodology via the Box-Behnken design using Digital expert Stat- Ease -360 software. HPTLC determination was carried out using Camag Linomet with densitometric scanner. The methods were verified as per ICH requirements, the stability of the drugs was considered under a forced stress environment, and observed for transitional degradation pattern. **Results:** The drug exhibits HPLC and HPTLC chromatographic peaks with retention periods of 6.18 and 5.61 min respectively with a clear and distinct peak for Voclosporin detected at wavelength maximums of 248 nm. The medication showed a percentage recovery ranging from 97% to 100% w/w. **Conclusion:** The developed method was found to be consistent and appropriate for repetitive screening of drugs in bulk dosage form as well as pharmaceutical formulation.

Keywords: HPLC, HPTLC, Multiple Sclerosis, Quality by Design, UV, Voclosporin.

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INTRODUCTION

The calcineurin inhibitor Voclosporin (VSN) has emerged as a broachable therapeutic option in the treatment of lupus nephritis. It may reduce the severity of this debilitating disease and improve clinical outcomes (Rovin *et al.*, 2021; Gelder *et al.*, 2022). Several elements of the adaptive immune system interact in a complex manner leading to the pathophysiology underlying lupus nephritis. Early diagnosis and management are important for patients in order to provide the best outcome. Thus, the identification of new biomarkers may enhance the therapeutic management and drug testing for lupus nephritis (Saxena *et al.*, 2024).

Numerous clinical trials have shown that VSN, a structural analog of the immunosuppressant cyclosporine, showed excellent efficacy as an anti-lupus nephritis agent. It has a superior pharmacokinetic profile compared to conventional calcineurin

inhibitors, possibly with fewer side effects. Induction and maintenance of remission, as an important outcome measure in clinical trials or observational studies, is one of the benefits measured on VSN based on current evidence with relevance to lupus nephritis treatment, it takes a follow-up biopsy to document if remission has truly occurred (Handy *et al.*, 2008).

QbD approach has a following benefit such as enhancing confidence in the quality of the product, well-defined regulatory flexibility and continuous improvement which can be achieved through understanding both "the Product" to be generated while taking account of different "Processes". QbD is a science- and risk-based Comprehensive approach that encompasses the principles of ICH Q8 Pharmaceutical Development, ICH Q9 Quality Risk Management, and ICH Q10 Pharmaceutical Quality System. The progress of a range of different scientific fields such as alimentary, pharmaceutical and earth sciences from this perspective is usually tied with the development of analytical techniques like chromatography or spectrophotometry (Lemaitre *et al.*, 2021; Carr *et al.*, 2014).

These analytical methods make a way to accompany and provide justification of the pharmaceuticals during production after which making those products have no longer been launched for sale except in conformity with relevant standards. Method validation



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is an essential step in method development, to demonstrate that the analytical methods are reliable, repeatable and capable of detecting changes in the quality attributes between analyte products. DOE is a powerful experimental design technique for improving and developing analytical methods. This helps scientists to systematically experiment with how the different parameters affect the outcomes of this process (Lemaitre *et al.*, 2020; Roberts *et al.*, 2008).

Several factors like pH, flow rate, column temperature and mobile phase composition also affect the efficiency of HPLC-based procedures. Response Surface Methodology (RSM) was employed to optimize the variables of analytical performances for better performance (Dall Era *et al.*, 2019). The box-Behnken plan was used, as it allows for a lesser no. of experimental runs to optimize the variables. A similar study has been carried out for the optimization of flow rate, mobile phase composition and pH (HPLC method) to separate and quantify drugs employing Box-Behnken design through RSM (Sun *et al.*, 2022).

This strategy aims to develop novel HPLC, HPTLC, and UV analytical procedures in conjunction with Box Behnken Design response surface methodology to guarantee the safety and efficacy of the medications in pharmaceutical dose form. Furthermore, it is crucial to develop the drug's analytical process, which is crucial for biomedical analysis (Koneru *et al.*, 2008).

MATERIALS AND METHODS

Instrumentation

Analytical Balance (WENSAR, HPB201, New Delhi, India), PVDF syringe filters with a pore size of 0.22 μm (Millex SLVVR33RS, Darmstadt, Germany). HPLC (Agilent 1260 Infinity G1322, Poway, USA), Double Beam Spectrophotometer (Cary 5000 Version 2.24, United States), HPTLC with Scanner (Camag 028.2000), Automatic Sample Applicator (Camag Linomat ATS 4, Mumbai), Precoated plate (RP-18, 10x10), Digital Expert Stat-ease 360 Software were used in present work.

Drugs and Reagents

Voclosporin reference standard (Alkem Laboratories, Kerala, India); HPLC-Grade Acetonitrile, Methanol and Water (Merck, India); Dihydrogen Orthophosphate, Formic acid and NaOH (Alpha Chemica, Delhi, India), Lupkynis Capsule (Aurinia Pharmaceuticals, Victoria, Canada).

GENERAL PROCEDURES

Preparation of Stock and Sample Solution

Pipette exactly enough stock solution to give a 10 mcg/mL concentration of VCN standard, and prepare a standard solution (solution A) with a concentration of 1 mg/mL using reference standard (Oliveira-Santos *et al.*, 2016). Each Lupkynis capsule (which contains 7.9 mg of Voclosporin) was weighed on average

and a glass stopper flask was filled with powder that contained exactly 100 mg of Voclosporin. Following a 10-min sonication process using 15 mL of methanol, the powder was extracted and subsequently filtered using Whatman No. 1 filter paper into a 100-mL standard flask. To achieve the desired volume, 10 μg /mL of drug solution was prepared by pipetting exactly the right amount of the previously described solution with methanol (Hall *et al.*, 2014).

Optimization of Spectral Characters

After the first 30-min stabilization period, alcohol was used to correct the blank in the absorbance spectra of the standard and test solutions of VSN, which were recorded in the 400-200 nm region. Using the UV-visible spectrophotometer, the scanning was done in spectra mode and the molar absorptivity of drug was determined (Hall *et al.*, 2014).

HPLC METHOD DEVELOPMENT

Chemometric-Based Factorial Design

Design optimization includes Critical Quality Attributes (CQA) and the Quality Target Product Profile (QTPP). CQA is used to identify the factors that impact the QTPP, while QTPP selects variables for investigation. In order to maintain the appropriate response range, it was necessary to regulate the following important parameters: pH, flow rate, and composition of the mobile phase. The retention duration was chosen by QTPP as the response. Using Design Expert® (Version 11.0, Stat-Ease Inc.) with quadratic response and square root transform, a statistical screening design was used to optimize and select key components. The several interaction effects and quadratic effects of selected factors on the retention time were examined.

Until the replies fall within the allowed ranges, the variable must be tuned at various levels. Design Expert tools will be used to optimize the appropriate chromatographic conditions.

Optimization of Mobile phase and Development of Chromatogram

HPLC's mobile phase was selected via a trial-and-error process combined with Box Behnken Design (BBD) based optimization, making it particularly suitable for optimizing chromatographic methods where extreme settings (e.g., very low pH or very high flow rate) may damage the column or compromise data quality. After mobile phase optimization, the entrapped air was removed by sonication and filtration through a 0.45 μm membrane filter while under vacuum. The trapped air was released by purging the mobile phase that had filled the reservoir. Following the stabilization of the baseline, 20 μL of analyte was injected, and the chromatogram was recorded for 15 min at 246 nm. The solvent was then allowed to run through the column at a flow rate of 1.0 mL/min. Each of the created VSN dilutions underwent the same

procedure twice. It was noted what the Area Under the Curve (AUC) and retention time were (Zakeri-Milani *et al.*, 2015).

HPTLC METHOD DEVELOPMENT

Methanol was used to prewash the TLC plate prior to Mobile Phase Selection, and it was activated for 5 min at 60°C in a hot air oven. The standard stock solution of VSN (100 ng/spot) was individually spotted on a TLC plate using a glass capillary tube, and it was then left to dry at room temperature for four to 5 min (Nijhu *et al.*, 2014). The mobile phase was placed in a CAMAG glass chamber and left for 20 min to saturate. Following the saturation phase, the spotted plate was left to continue developing in the saturated mobile phase. After that, the plate was dried and was gently taken out of the glass container. For band tailing, band form, band separation, and other purposes, spots were perceived in the CAMAG TLC Visualizer (Maher, 2014).

To get an excellent resolution and an acceptable R_f value, several trials were carried out by varying the composition and ratio of the mobile phase. After several trials with various reagents, the optimal mobile phase was chosen.

To ascertain the quantity of VSN in the formulation, A 0.1 μ L supernatant layer of sample solution (equivalent to 100 ng/spot of VSN) was placed onto an HPTLC plate using the solution. The plate was processed and imaged. Three replicas of the analysis were conducted. Relative responses to the VSN regression line equation were used to calculate the content of drug in the formulation. The drug's percentage assay was then computed.

Optimization of Developed Methods

Specificity

Developed Methods were followed for the analysis of placebo and blank interference. Following injection under the previously specified chromatographic conditions, chromatograms for the blank and placebo were recorded (Moharana *et al.*, 2019).

Linearity

While plotting concentration versus detector response a linear pattern of area was observed for VSN. An evaluation was conducted on the correlation between concentration and the area response coefficient of determination (Gatkal *et al.*, 2014).

Accuracy

The accuracy of this test procedure was tested by generating recovery samples of VSN at 20%, 100%, and 150% of the planned stock sample concentration level. There were three retrieval samples created for every concentration level. Following the aforementioned samples' recording, the percentage recovery of each sample was calculated for the quantity added (Kumar *et al.*, 2019).

Stability

A range of conditions, including bench top at ambient temperature and in a refrigerator at 2 to 8°C, were used to prepare the standard and sample solutions for solution stability. The stability of both the standard and sample solutions were determined by comparing the initially prepared solutions with the most recently prepared ones (Teuta *et al.*, 2024).

Precision

Conducting a technique precision investigation across six preparations of the same sample confirmed the approach's intermediate accuracy on separate days, representing the same batch. According to the protocol, these samples were analyzed (Azmi *et al.*, 2024).

LOD and LOQ

Following a regression equation analysis of the one-dimensional data, the anticipated detection and quantitation limit was determined along with its slope and standard deviation (Moharana *et al.*, 2011).

Statistical Analysis

Statistical evaluation of the experimental data was carried out using Design Expert® software (Stat-Ease Inc., Version 11.0). A Box-Behnken Design (BBD) with three independent variables was employed to study their individual and interaction effects on the chromatographic response. Analysis of Variance (ANOVA) was used to determine the significance of the model, main factors, interaction terms, and quadratic terms. Model adequacy was assessed based on F-values, p -values (<0.05 considered significant), Adjusted R^2 , Predicted R^2 , and lack-of-fit tests. Response Surface Plots and Contour Plots were generated to visualize factor interactions and to identify optimal chromatographic conditions.

For method validation, linear regression analysis was used to calculate slope, intercept, correlation coefficient (R^2), and to estimate LOD and LOQ from the standard deviation of the response and slope of the calibration curve. Precision, accuracy, and stability results were expressed as mean $\pm$ Standard Deviation (SD) and % Relative Standard Deviation (%RSD). All measurements were performed in triplicate to ensure reproducibility and statistical reliability.

Ethical Statement

This study did not involve human participants or animal subjects; therefore, ethical approval was not required.

RESULTS

VSN standard curves in methanol were found to be linear in the concentration range of 2-10 μ g/mL. To determine the wavelength of maximum absorption (λ_{max}), a scan of the drug solution in

the UV region (200-400 nm) was conducted. At 248 nm, the wavelength maximum was observed. Figure 1 displays the UV spectrum (Chatki *et al.*, 2024).

HPLC METHOD DEVELOPMENT

The critical optimization factors selected for the Box-Behnken Design pH, organic phase modifier volume, and flow rate along with their experimental ranges, are listed in Table 1 and the optimization parameter for Quadratic model epitomized in Table 2.

Response Surface Plots (RSP) and Contour Plot (CP)

The quadratic equation is represented graphically by RSP and CP, which are used to evaluate the interaction between factors and their impact on the projected response (Retention Time). A correlation between the selected factors and the percentage recovery was found by the use of multi-regression analysis on the collected data. In Figure 2, the contour plot and 3D surface design based on the estimated reaction are displayed (Azmi *et al.*, 2024).

Response surface design was used at the low-high level to enhance the performance of the HPLC process. Surface characterization using Box- Behnken design employing a quadratic equation was used to examine the different interactions and quadratic effects of the organic modifiers, pH, and flow rate within a given range (low-high). Table 3 included the experimental runs along with the corresponding responses for the quadratic ANOVA model and retention time for the quadratic model (Gullapalli *et al.*, 2024).

Given that the model's F-value was determined to be 17.75, it is likely to be significant. An F-value of this high value could only be

the result of noise in 0.05% of cases. Model terms are significant if the *p*-value is less than 0.0500. Here, the model terms C and C² are important. The model terms are deemed not significant if their values exceed 0.1000. It is implied that the lack of fit is substantial by the F-value of 18.08. A big Lack of Fit F-value has a very slim probability of occurring because of noise (0.86%). Based on the observations, the final VSN retention time equation using coded factors was developed (Gullapalli *et al.*, 2024).

Retention time of VSN= $14.41587+0.535362A-0.242162B-2.89706C+0.005426BC+0.028076AC+0.004837A^2+0.002909B^2+0.210136C^2$

Where A represents the flow rate, B is the quantity of organic phase modifier, and C is the pH with a retention time of 6.18, the improved mobile phase showed consistent peak shape and repeatable findings at ratios of 0.05 M KH₂PO₄: Acetonitrile-70: 30 at pH 6.8 and flow rate of 1 mL/min. Figure 3 displayed the chromatogram in relation to retention duration (Elkady *et al.*, 2024).

HPTLC METHOD DEVELOPMENT

Using the linear ascending development technique, many experiments were conducted with varying solvent contents for the mobile phase development. For VSN, the optimized mobile phase with the ratio of Methanol to Water to Formic acid (4: 5: 1 v/v/v) demonstrated a good resolution at R_f 0.57. Densitometric data were acquired using the vision CATS software-controlled Camag TLC scanner IV at 254 nm. Figure 3 demonstrates the chromatogram of VSN with a Retention time of 0.57 min (Nakov *et al.*, 2015).

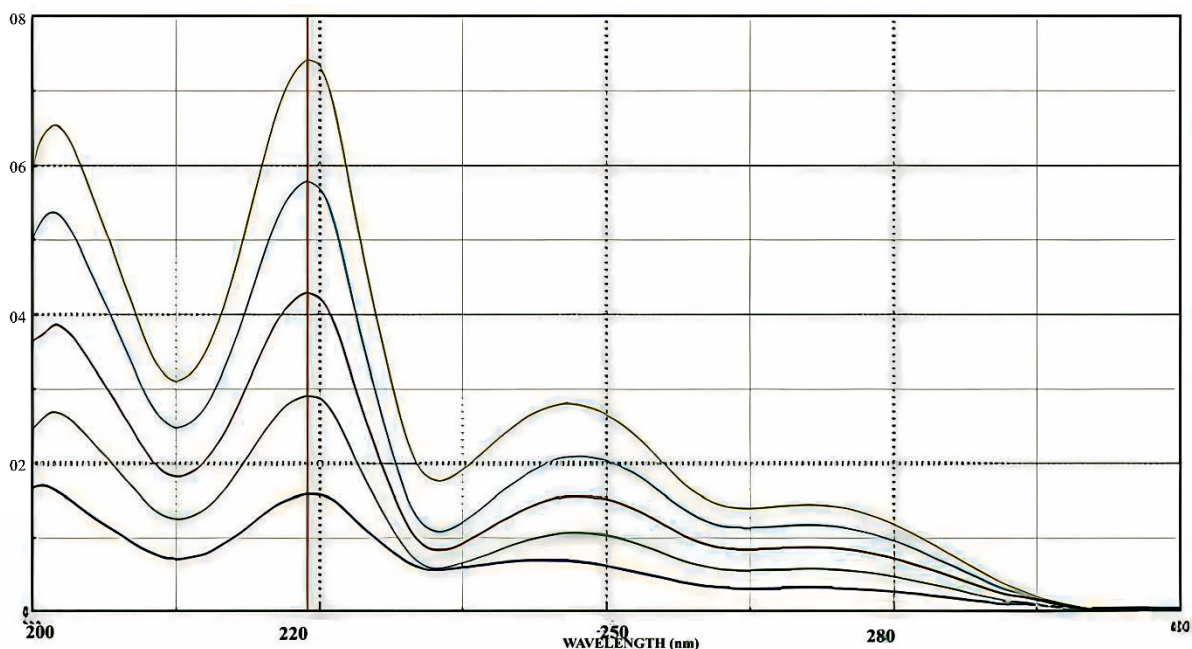


Figure 1: UV spectra of VCN.

Table 1: Optimization Factors for Experimental Design in HPLC.

Factors	Name	Units	Minimum	Maximum	Mean	Std. Dev
A	pH	-	6.50	7.50	7.00	0.3536
B	Organic Phase Modifier	mL	10.00	20.00	20.00	7.07
C	Flow Rate	mL/min	0.7500	1.25	1.0000	0.1768

Table 2: Optimization Parameter for Quadratic Model.

Source	Sequential <i>p</i> -value	Lack of Fit <i>p</i> -value	Adjusted R ²	Predicted R ²	
Linear	0.0026	0.0008	0.5725	0.3488	
2FI	0.9879	0.0004	0.4511	-0.4470	
Quadratic	0.0014	0.0086	0.9041	0.3700	Suggested
Cubic	0.0086		0.9885		Aliased

Table 3: ANOVA for Quadratic Model considering coded Factors for Optimization of Mobile phase.

Retention Time Response by Square Root Transform					Lack of fit summary			
	Factor A	Factor B	Factor C	Response	Source	Sum of Squares	F-value	<i>p</i> -value
Std Run	Flow Rate (mL/min)	Organic Phase (mL)	pH	Retention time				
1	0.75	30	7	7.56	Model	0.7096	17.75	0.0005
2	1.5	30	7	7.56	Factor A	0.00002	0.0001	0.9942
3	1	20	6	6.18	Factor B	0.0004	0.0884	0.7749
4	1.5	40	7	7.56	Factor C	0.4830	108.74	<0.0001
5	1	20	6	6.18	AB	0.0004	0.0932	0.7690
6	1.5	35	6	7.36	AC	0.0004	0.0998	0.7613
7	0.75	35	8	12.45	BC	0.0023	0.5267	0.4916
8	1.5	35	8	12.45	A ²	0.0140	3.15	0.1192
9	1.125	20	6	6.18	B ²	0.0223	5.01	0.0602
10	1.125	25	6	6.38	C ²	0.1859	41.86	0.0003
11	1.125	30	8	13.1	Residual	0.0311		
12	1.125	40	8	13.45	Lack of Fit	0.0290	18.08	0.0086

Optimization of Analytical Results

There was no peak visible on the blank solution chromatogram during the VSN peak's retention period. This suggests that the blank solution utilized in the fabrication of the standard and sample does not affect the estimate of VSN in the formulation of tablets settles the specificity (Nakov *et al.*, 2015).

A recovery study utilizing the standard addition technique was conducted at three different levels (80%, 100%, and 120%) to assess accuracy. Over 95% of the recovered percentage was found, with a relative standard deviation of less than 2. Concerning the UV radiation concentration and absorbance, the curve exhibits a linear relationship at concentrations between 5 and 30 mcg/

mL; likewise, the peak area vs. concentration curve shows a linear relationship between 5 and 50 mcg/mL (HPLC) and 50-250 ng/spot (HPTLC) (Chatki *et al.*, 2014).

The stability experiment results reveal a decrease in drug recovery percentage, which is accompanied by a decrease in drug UV absorbance in specific pressured situations. This suggests that pharmaceuticals are deteriorating abstemiously in stressed environments. With a pH range of 4-8, the drugs showed stability up to 80°C for 48 hr at room temperature and in a refrigerator (2-8°C) for 3 hr when exposed to light (Elsinghorst *et al.*, 2011).

The findings indicate that the UV technique yielded values larger than 1 µg/mL, while the HPLC and HPTLC methods yielded

values less than 1 µg/mL. With a linearity coefficient of 0.9800-0.9999, a strong association was discovered. Table 4 contains a complete set of the validation findings.

DISCUSSION

Maximum absorbance in the UV spectra of VSN is observed at wavelengths 248 nm. At concentrations ranging from 2 to 50 mcg/mL, these wavelengths follow Beer-Lambert's rule. With an RP-HPLC column and a non-polar mobile phase, the aforementioned drugs are separated at a flow rate of 1 mL/min and a pH of less than 8 and more than 4 (Nijhu *et al.*, 2011). The column's retention duration is 6.18 and its peak area extends from 2100000 to 2300000 at 100 µg/mL, since the medication under examination was polar and could be separated utilizing reverse-phase chromatography. Acetonitrile and buffer were combined to form the mobile phase, while a C₁₈ column served as the stationary phase. To get the right resolution, several ratios were then applied according to the BBD design (Yasmeen *et al.*, 2013).

The newly developed method, guided by a Quality by Design (QbD) approach using Box-Behnken Design (BBD), addresses these shortcomings by systematically optimizing critical method variables such as mobile phase composition, pH, and flow rate.

This ensures robust chromatographic performance with minimal variability, even under deliberately altered conditions. During the selection and optimization of the mobile phase, it was found that a greater proportion of organic phase shortens the time that pharmaceuticals are retained and makes the peak sharper, while a greater proportion of the aqueous phase makes the peak broader. The pH of the mobile phase was chosen to be at least two units lower than the pKa of the therapeutic ingredient. The drug's pKa varies from 3 to 8, thus pH was used as the buffer and the ratio of Acetonitrile to KH₂PO₄ was set to 70:30 for the composition of the mobile phase with a flow rate of 1 mL/min, pump pressure was 400 bar, and the stop time was set at 15 min. The devised approach yielded good resolution and an optimal retention time.

A satisfactory resolution was shown at R_f 0.57 by the optimized mobile phase with the ratio of Methanol to Water to Formic acid (4: 5: 1 v/v/v) in the HPTLC method, The peak area during densitometric analysis was shown to be between 5000 and 5700 for 100 ng/spot (Luo *et al.*, 2020). The procedure's dependability and the linearity of the study are supported by the HPLC, HPTLC, and UV methods with a regression equation with a coefficient of 0.9999, as well as by LOD and LOQ lying within the range.

Excipient interference was never seen throughout the method's development. The recovery percentage's low standard

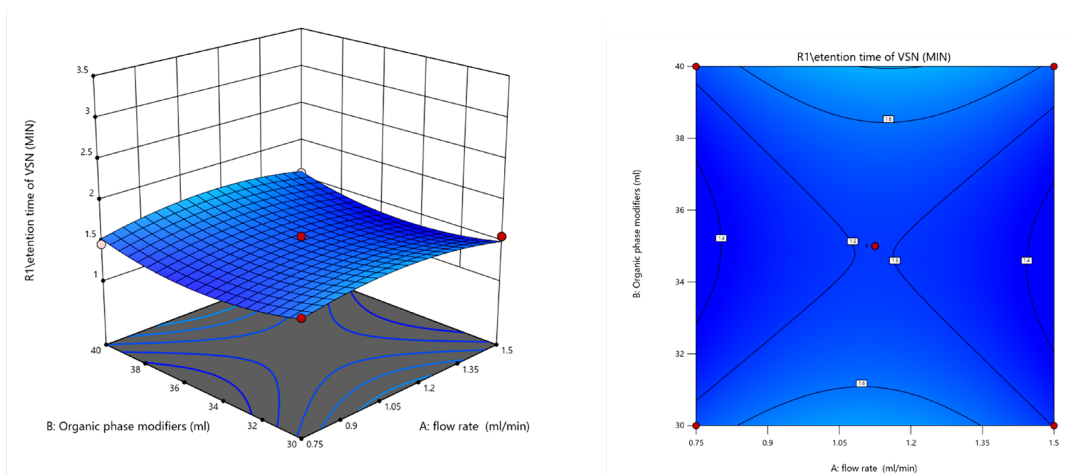


Figure 2: 3D Response Surface and 2D Contour Plot of VSN.

Table 4: Results of evaluation Parameters for Validation of VSN.

Parameters	UV	HPTLC	HPLC
Concentration	5-50 µg/mL	50-250 ng/spot	100-500 µg/mL
Intercept	0.0325	5231	3.1648
Slope	0.0056	0.2066	0.2785
RSD (%)	0.7456	0.0984	0.4165
Coefficient Of Variation	0.0007	0.0002	0.0041
Correlation Coefficient	0.9997	0.9956	0.9963
LOD (µg/mL)	4.5862	3.4215	1.2541
LOQ(µg/mL)	13.8975	6.2351	2.1253
Drug Recovery (%)	97.5900	96.8500	97.8600

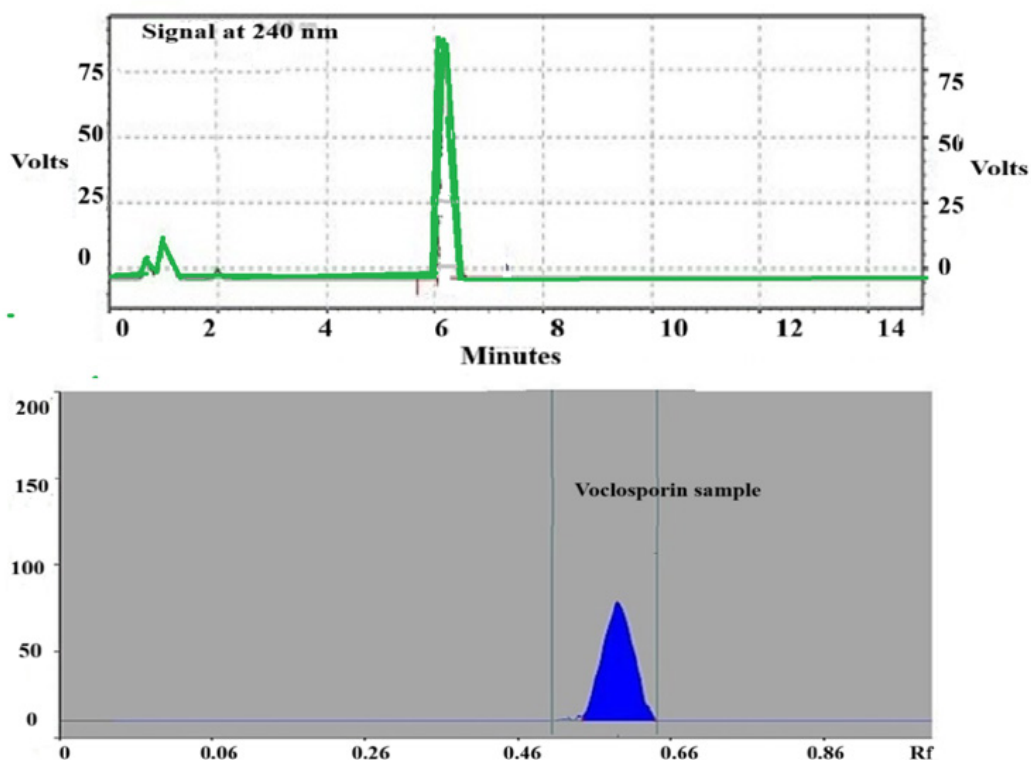


Figure 3: HPLC & HPTLC Chromatogram of VSN.

deviation provided additional proof of the method's reliability. Excipient interference was never seen throughout the method's development. The procedure's dependability and the linearity of the study are supported by the HPLC, HPTLC, and UV methods with a regression equation with a coefficient of 0.9999, as well as by LOD and LOQ lying within the range. The recovery percentage's low standard deviation provided additional proof of the method's reliability. Excipient interference was never seen throughout the method's development. The recovery percentage's low standard deviation provided additional proof of the method's reliability. Excipient interference was never seen throughout the method's development (Zakeri-Milani *et al.*, 2005). Hence, the findings demonstrate that the technique is also applicable to the routine examination of VSN in biomedical samples.

CONCLUSION

To quantitatively determine VSN, an isocratic, quick, easy, sensitive HPLC and HPTLC approach supporting UV spectroscopy was devised. The UV wavelength for detection was specified in each designed HPLC and HPTLC technique. The HPLC mobile phases consisted of different ratios of acetonitrile and phosphate buffer and the HPTLC mobile phase was Methanol: Water: Formic acid (4: 5: 1 v/v/v). Linearity ($r=0.999$) was reported in the observed range. Lower and higher values impact the % recovery owing to variations in flow rate, pH, and organic phase. Box Behnken validation indicates stability. Temperature, hydrolysis, and oxidation are stressors that lower the recovery percentage. It

demonstrates that the created approach is appropriate for regular VSN screening in pharmaceutical formulation. The developed HPLC method is highly suitable for its intended matrix, which is the pharmaceutical formulation of Voclosporin. During method validation, the assay demonstrated excellent specificity, showing no interference from common excipients or degradation products, confirming its suitability for routine quality control in tablet or capsule dosage forms.

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ABBREVIATIONS

VSN: Voclosporin; **UV:** Ultra Violet; **HPLC:** High-Performance Liquid Chromatography; **HPTLC:** High-Performance Thin Layer Chromatography; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **RSD:** Relative Standard Deviation; **MS:** Multiple Sclerosis; **BBD:** Box Behnken Design; **RSP:** Response Surface Plot; **CP:** Contour Plot; **CQA:** Critical Quality Attributes; **QTPP:** Quality Target Product Profile. QbD: Quality by Design; ICH: International Council for Harmonisation; DOE: Design of Experiments; RSM: Response Surface Methodology; AUC: Area under the Curve; ANOVA: Analysis of Variance.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

Hamza Kutty Pottengal performed the experimental work, data analysis, and manuscript drafting. Prasanth Viswanadhan Vasantha and Rakeshkumar Jat supervised the research and contributed to method optimization and manuscript revision.

SUMMARY

The study developed and optimized UV, HPLC, and HPTLC methods for quantifying Voclosporin using a QbD-based Box-Behnken design. Key parameters were optimized to achieve reliable chromatographic performance. The methods showed good linearity, precision, accuracy, and stability, making them suitable for routine quality control of Voclosporin formulations.

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