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Development of Robust RP-HPLC Method for Posaconazole Assay in Liposomal Formulation using QbD and ICH-Guided Validation with Forced Degradation Analysis

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Posaconazole, an antifungal for invasive infections, requires liposomal formulation to overcome poor solubility and bioavailability, making robust analytical methods essential to ensure quality despite interference from excipients. The aim was to develop and validate a reverse-phase HPLC method for the quantification of posaconazole in a liposomal formulation, utilizing Design Expert software. This method was developed to ensure consistency and reliability as per the ICH guidelines. Chromatographic separation was carried out using a reverse-phase HPLC system assembled with a C_{18} (ODS) column (150 × 4.6 mm, 3.5 µm). A mixture of methanol and buffer in a 70:30 (v/v) ratio served as the mobile phase, with a flow rate maintained at 1 mL/min. A Quality by design (QbD) approach was employed to optimize key chromatographic conditions, including the ratio of the mobile phases, pH and rate of mobile phase flow. The validation of the developed method was done based on linearity, accuracy, precision, sensitivity, specificity and robustness. The optimized RP-HPLC method showed a linear response with a correlation coefficient (r^2) of 0.9994 across the concentration range of 800-1200 µg/mL, ensuring accurate quantification of posaconazole in liposomal formulation. Precision studies demonstrated low relative standard deviations (RSD), with intra-day precision of 0.12% and inter-day precision ranging from 0.36% to 0.39%. The accuracy of the method was 98.4% to 99.4%, confirming its reliability for routine use. Ruggedness testing yielded consistent results from two analysts, with RSD values of 1.38% and 1.42%, indicating the reliability and robustness of the method. Specificity testing confirmed that the method can accurately quantify posaconazole in the presence of formulation excipients, with no interference observed. Furthermore, forced degradation stability studies revealed the stability-indicating ability of the method in terms of a clear distinction between the drug and subsequent degradation products. Based on results, this validated method provides a reliable tool for ensuring the consistent quality and therapeutic efficacy of posaconazole liposomal formulation.

Keywords: Posaconazole, Liposomes, RP-HPLC, Optimization, Validation.

INTRODUCTION

Posaconazole, a second-generation triazole antifungal, offers broad-spectrum activity and is widely used for prophylaxis and treatment of severe invasive fungal infections, particularly in immunocompromised patients such as those undergoing chemotherapy, stem cell transplants or living with HIV/AIDS [1]. By inhibiting fungal cell membrane synthesis, posaconazole plays a vital role in antifungal therapy and is essential in protecting vulnerable individuals from potentially life-threatening infections [1,2]. It works by specifically targeting and inhibiting lanosterol 14α-demethylase, a cytochrome P450 enzyme essential for the formation of ergosterol, a key sterol in fungal cell membranes. Inhibiting this enzyme disrupts ergosterol synthesis, thereby compromising the integrity and functionality of the fungal cell membrane. This disruption compromises membrane integrity, ultimately causing cellular damage and death in the fungal organism. The targeted inhibition of this enzyme is central to its antifungal effectiveness and therapeutic action against fungal infections [3,4]. Despite its therapeutic efficacy, posaconazole exhibits poor aqueous solubility and a pH-dependent dissolution profile, which severely limits its oral bioavailability and poses challenges in achieving consistent systemic exposure [5,6].

To overcome these pharmacokinetic limitations, liposomal formulation strategies have emerged as promising alternatives. Poorly water-soluble drugs such as posaconazole can exhibit enhanced dissolution rates and improved bioavailability

when delivered through nanocarrier systems like nanocrystals, liposomes, micelles and polymer-based nanoparticles, owing to their ability to increase surface area and facilitate more effective interaction with the gastrointestinal environment [7,8]. However, the accurate quantification of posaconazole in such liposomal drug products is analytically challenging due to the complex formulation matrices, potential excipient interferences and variable drug-polymer interactions.

Although advanced techniques like LC-MS/MS are available for posaconazole quantification in biological matrices, they are often resource-intensive, costly and inaccessible for routine quality control purposes in many pharmaceutical settings [9,10]. In contrast, reverse-phase high-performance liquid chromatography offers a more practical, cost-effective and widely available alternative for drug estimation, especially in formulation development and routine analysis [11].

In this regard, the current research focuses on establishing a reliable, accurate and reproducible reverse-phase HPLC method for quantifying posaconazole within a liposomal formulation, guided by the quality by design (QbD) approach. This approach leverages design of experiments (DoE) to systematically develop the method, identify critical method parameters (CMPs) and assess their interactions, thereby enhancing robustness, performance and compliance with regulatory standards. The proposed method, validated in accordance with ICH Q2(R1) guidelines, fulfills key criteria and incorporates forced degradation studies to establish its stability-indicating ability for reliable quality control of liposomal posaconazole.

EXPERIMENTAL

The reference standard of posaconazole with a certified purity of 99.5% was procured from Sigma-Aldrich, ensuring high analytical reliability for calibration and quantification. All other chemicals and solvents required for the formulation and analytical procedures, including excipients and degradation inducing agents, were also sourced from Sigma-Aldrich, maintaining consistency and reagent quality throughout the experimental work. HPLC-grade methanol, used as a mobile phase, was purchased from Merck Life Sciences to ensure the optimal chromatographic performance and minimal baseline noise. Analytical-grade reagents were employed wherever applicable for sample preparation, analysis and method validation. Additionally, Milli-Q water was used throughout the study for dilution, solution preparation and system suitability evaluations, thereby eliminating contamination risk and guaranteeing the reproducibility of results. These high-purity materials underpin the robustness and reliability of the RP-HPLC method developed and validated in this work.

Instrumentation: RP-HPLC analysis was conducted using the Agilent 1200 Infinity HPLC system, which was equipped with a UV-visible detector. Chromatographic analysis was performed on a C_{18} ODS column (150 × 4.6 mm, 3.5 μm) using a binary gradient mobile phase comprising methanol and buffer in a 70:30 (v/v) ratio. The process of data collection and analysis was conducted with the help of EZ Chrom Elite software. The analysis was conducted at a column temperature maintained at 40 ºC under ambient conditions, ensuring optimal separation performance. This setup provided effective resolution of the analytes while maintaining consistent temperature control throughout the process for reliable chromatographic results. All samples weighing procedures were executed using an INCAL analytical balance provided by Scale-Tec, ensuring precision in the preparation steps. The detection of the analyte was carried out at a specific wavelength identified through preliminary UV-visible spectral analysis using a Perkin-Elmer Lambda 35 spectrophotometer. This wavelength was determined after scanning a standard solution of posaconazole to optimize detection parameters. A 5 μL aliquot of the prepared sample was injected into the HPLC system for analysis. The total runtime for each chromatographic run was 10 min, allowing efficient and rapid assessment of the sample components. This method ensured reliable separation and detection suitable for the intended analytical evaluation [12].

Stock solution of standard drug: To prepare the primary stock solution of posaconazole, a solvent system composed of methanol and buffer in equal proportions (1:1, v/v) was utilized. The buffer was formulated by dissolving 1.74 g of dipotassium hydrogen phosphate in 1000 mL of distilled water, followed by careful pH adjustment to 1.7 using orthophosphoric acid. Subsequently, an accurately weighed amount of 10 mg of finely ground posaconazole was transferred into a 100 mL volumetric flask for solution preparation. The drug was then solubilized by mixing with the prepared methanol-buffer solution and the volume was made up to 100 mL mark using the same solvent mixture. To ensure clarity and eliminate any undissolved particles, the prepared solution was filtered through a membrane filter with a 0.45 μm pore size (Millex®, Sigma-Aldrich, USA). An appropriate volume of the clear filtrate was then taken and further diluted using the methanol-buffer mixture to prepare a working solution. The final concentration of this diluted solution was adjusted to 100 μg/mL, suitable for subsequent analytical or experimental use. This approach ensured the reproducibility and consistency required for accurate quantitative analysis involving posaconazole. This preparation ensured uniformity and clarity in the solution, suitable for further analytical procedures. The described method provides a reliable and consistent approach to formulating a standard solution of posaconazole, maintaining accuracy in concentration and stability for analytical applications.

Stock solution of drug liposomal formulation: To prepare the solution, the liposomal formulation of posaconazole was initially mixed with 100 mL of 1:1 methanol-to-buffer solution. This process yielded a final drug concentration of 100 μg/mL. The resulting mixture was then passed through a 0.45 μm membrane filter (Millex®, Sigma-Aldrich, USA) to remove particulate impurities. After filtration, a suitable volume of the solution was taken and diluted again using the same methanol-buffer mixture in order to preserve the drug concentration at 100 μg/mL, ensuring consistency and accuracy for further analysis or use in experiments. This process ensured the preparation of a clean, accurately diluted sample for further analysis.

Optimization of RP-HPLC method: Initially, a trial-and-error approach was adopted to gain preliminary insights into the process efficiency and to identify key independent variables influencing the outcomes. This exploratory step helped determine the factors with notable effects on the selected

responses. The experimental optimization was carried out using Design Expert® Software (version 13, Stat-Ease Inc., Minneapolis, USA), applying a Box-Behnken design (BBD) framework. This design involved three independent variables methanol concentration (X1, 30-70%), pH level (X2, 1 to 2) and flow rate (X3, 0.5 to 1.5mL/min), each tested at three distinct levels. The performance of the process was evaluated through two dependent outcomes: retention time (Y1) and the number of theoretical plates (Y2). This statistical approach enabled the systematic analysis of the variables' effects and their interactions to enhance chromatographic performance under optimized experimental conditions. This statistical design enabled a systematic evaluation of how the variations in these three independent factors influenced the selected responses, thus facilitating the development of an optimized set of conditions for the analytical method. The use of BBD ensured minimal experimental runs while providing reliable and reproducible data for analysis. An experimental approach was adopted in the study, wherein the independent variables were evaluated at three distinct levels: low (–1), medium (0) and high (+1). The design software recommended a total of 15 experimental runs, which included three replicates at the central point to improve statistical accuracy. To maintain the validity and consistency of the outcomes, all other formulation and process-related parameters were held constant throughout the experimentation. To enhance statistical robustness, three additional center point replicates were incorporated into the experimental set. All non-variable parameters were maintained at their optimal values throughout the study. The DoE software was configured to evaluate retention time and theoretical plate, excluding any influence from the independent variables. Response surface methodology (RSM) was employed to predict optimal levels of the independent variables, understand their interactions and develop a suitable analytical method. The reliability and importance of the model were evaluated using Analysis of Variance (ANOVA). To identify the optimal experimental conditions, a desirability function was applied alongside checkpoint analysis, based on predefined target values for critical quality parameters. Based on the optimization criteria provided by the software, a single set of HPLC conditions was identified and tested in triplicate to validate the method's performance [13]. Model validation was performed by analyzing the correlation between predicted and experimental values of retention time and theoretical plates.

Validation of predicted point and data analysis: The obtained data were analyzed using Design-Expert software developed by Stat-Ease. Multiple linear regression analysis (MLRA) was used to evaluate the influence of various independent variables on the response parameters. This analysis involved constructing second-order polynomial equations with the help of the software. The validity and reliability of the developed models were evaluated through ANOVA, which confirmed their statistical significance. Based on the optimization criteria set within the software, specific checkpoint formulations were suggested. These formulations were then prepared experimentally and their actual outcomes were measured. To ensure model accuracy, the experimental results were compared with the predicted values provided by the software. The close agreement between predicted and observed values validated the optimization process. Following this validation, the same optimized formulation was selected for further studies aimed at achieving the desired product characteristics. This systematic approach ensured that the experimental design was both predictive and reproducible, facilitating a robust formulation development process [14].

Validation of method

System suitability: To verify proper system performance, the system suitability parameters were evaluated. Precision was assessed using six replicate injections of 1000 ppm posaconazole standard solution. Key indicators like the theoretical plates and the tailing factor were examined as well.

Selectivity: A posaconazole liposomal formulation sample was prepared to evaluate the selectivity of the proposed method. The chromatographic area of the formulation was measured and compared with that of a standard solution. Percentage recovery of both analytes was also evaluated to validate the method's performance and ensure accurate quantification. This comparison confirmed the reliability and specificity of the analytical method for the liposomal sample, supporting its suitability for routine analysis of similar formulations.

Linearity: The linearity of the proposed analytical method was assessed following the ICH guidelines. Linearity reflects the ability of method to produce results directly proportional to the analyte concentration within a defined range. To assess this, posaconazole solutions were prepared at concentrations of 800, 900, 1000, 1100 and 1200 ppm. These solutions were used to generate a calibration curve for quantifying posaconazole in liposomal formulations. Each concentration level was injected independently into the HPLC system three times to ensure reproducibility. The peak areas corresponding to each concentration were then plotted against their respective concentrations to establish the calibration curve. The data generated was subjected to linear regression analysis to identify the best-fitting line that represents the relationship between posaconazole concentration and detector response. Moreover, the correlation coefficient (R^2) was computed to evaluate the accuracy and consistency of the linear association. This approach ensured that the analytical method reliably correlates the peak area with posaconazole concentration within the tested range, confirming the suitability of method for quantitative analysis in the given formulation. This step ensures that the analytical procedure maintains accuracy and precision in quantifying posaconazole within the specified range, thereby confirming the suitability of method for further use in pharmaceutical research and formulation studies.

Accuracy: The drug recovery process was carried out by injecting solutions (n = 6) containing predetermined concentrations (1000 ppm) of the two drugs, freshly prepared from stock solutions. Throughout the experiment, three different liposomal formulations were prepared with varying concentrations at 80%, 100% and 120%. The standard posaconazole solutions were prepared similarly at these three concentration levels. Each concentration was analyzed in triplicate using the HPLC technique. From the resulting data, the percentage of drug recovery was calculated, alongside the relative standard deviation for each concentration level. This approach ensured accurate evaluation of drug recovery across multiple formul-

ation strengths, providing insight into the consistency and reliability of the liposomal preparations and the analytical method employed.

Method precision: To evaluate the accuracy of the initial method, both intra-day and inter-day precision assessments were performed. For intra-day precision, six repeated injections of 1000 ppm sample solutions were analyzed using the validated HPLC technique. These injections were conducted twice within the same day, once in the morning and again in the evening, to check for consistency in results during a single day. The inter-day precision was assessed by performing six replicate injections on three separate days: six injections on the morning of the first day, followed by six injections each on the mornings of the subsequent two days. This approach ensured that variability over multiple days was captured. The precision of the method was then evaluated by analyzing the peak area data from these injections. The extent of variability was measured by calculating the relative standard deviation (RSD), also known as the percentage coefficient of variation (CV). These statistical measures provided a clear indication of the method's reliability and reproducibility for the sample concentrations tested.

Robustness: The ICH defines the robustness of an analytical method as its ability to withstand small, intentional changes in method parameters without being affected. In this robustness evaluation, factors such as buffer pH, the composition of mobile phase, injection volume, flow rate and the temperature of column oven were examined. To assess these variables, posaconazole samples at 1000 ppm were injected three times under each modified condition using the validated HPLC procedure. The robustness of method was evaluated by determining the percentage coefficient of variation (%CV), a measure that reflects its precision and consistency even when key operational conditions are intentionally altered.

Forced degradation study: Forced degradation studies were performed on 1000 ppm posaconazole to evaluate its stability under various stress conditions, including acidic, basic, oxidative, thermal and photolytic conditions, providing comprehensive insight into its degradation behaviour. For acid hydrolysis, 25 mg of posaconazole was treated with 0.1 M and 1.0 M HCl (5 mL and 10 mL) for 1 h at room temperature, followed by neutralization with equimolar NaOH and dilution to 25 mL with mobile phase [15]. In base hydrolysis, 25 mg of drug was exposed to 0.1 M and 1.0 M NaOH (5 mL and 10 mL) for 1 h, then neutralized with equimolar HCl and diluted similarly [16]. Oxidative degradation was induced by treating 25 mg of posaconazole with 10% H_2O_2 (5 mL and 10 mL) at ambient temperature for 1 h, followed by dilution without neutralization due to minimal pH change [16]. For thermal degradation, 25 mg of drug was exposed to 70 ºC in a hot air oven for 24 h, then dissolved and diluted with mobile phase [17]. Photolytic degradation involved UV exposure at 245 nm for 24 h before dilution to 25 mL with mobile phase [17]. All stressed and control samples were analyzed *via* a validated HPLC method, and degradation was assessed by comparing peak areas to the untreated drug.

Statistical analysis: Data is presented as the mean SD, with n = 6.

RESULTS AND DISCUSSION

Optimized RP-HPLC method: Using response surface methodology approach, the RP-HPLC method was statistically optimize for posaconazole estimation in liposomal formulation. Utilizing specialized software, polynomial equations were generated alongside contour and 3D response surface plots, facilitating a comprehensive multi-level regression analysis. The observed retention times ranged from 3.92 to 4.86 min, while the number of theoretical plates varied between 13,580 and 14,954 indicating system efficiency. The UV-visible spectrophotometric evaluation identified the maximum absorbance wavelength for posaconazole at 262 nm, as depicted in Fig. 1. Table-1 presents detailed response data from all experimental batches, summarizing the outcomes of various trials conducted to optimize and validate the method parameters. This approach

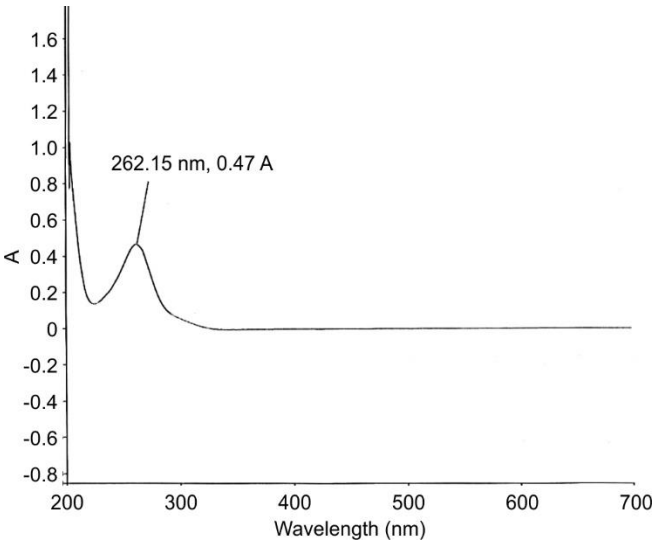

Fig. 1. UV-visible spectrum of posaconazole

TABLE-1
EXPERIMENTAL RUNS FOR DEPICTING THE EFFECT OF METHANOL COMPOSITION, PH AND FLOW RATE ON RETENTION TIME AND THEORETICAL PLATES IN HPLC ANALYSIS

Runs	Independent variables			Retention time	Theoretical plates
	Composition of methanol (%)	pH	flow rate		
1	40	1.5	1.0	4.25	14008
2	40	2.0	0.5	4.82	14879
3	40	1.0	0.5	4.86	13971
4	30	1.5	0.5	4.36	13892
5	40	1.0	1.5	4.45	13976
6	40	2.0	1.5	4.56	14954
7	50	1.0	1.0	4.32	14003
8	50	1.5	1.5	4.85	13913
9	40	1.5	1.0	4.25	13979
10	50	2.0	1.0	3.98	14431
11	40	1.5	1.0	4.50	13918
12	30	1.5	1.5	3.99	13854
13	50	1.5	0.5	4.55	13801
14	30	2.0	1.0	3.92	14901
15	30	1.0	1.0	4.86	13580

offered a robust statistical framework to evaluate the impact of formulation variables on chromatographic performance and the overall quality of the liposomal posaconazole system.

Effect of independent parameters on retention time: The model produced a polynomial equation for response Y1 (retention time), where the factor coefficients reveal the key individual and interaction effects impacting the response. By using an equation that incorporates coded factors, it becomes possible to predict the outcome based on specific factor levels. Typically, higher factor levels are represented by +1, while lower levels are denoted by -1. This approach enables clear interpretation of factor effects on retention time, supporting effective optimization and control within the experimental setup [18].

$$\text{Retention time (Y1)} = 6.693 - 0.684\,X1 + 0.377X2 + 0.382X3 \quad (1)$$

The equation indicates that pH (X2) and flow rate (X3) have a positive effect on retention time, while methanol content (X1) has a negative effect. Increasing pH and flow rate from low to moderate levels leads to longer retention times, whereas reducing methanol content below its optimal level also increases retention time. Thus, retention time is extended by elevating pH and flow rate within a certain range or by decreasing methanol concentration below its ideal point.

Interaction effect on theoretical plates: Eqn. 2 reveals a clear positive correlation between the pH level and the number of theoretical plates. The results indicate that increasing both the pH (X2) and the flow rate (X3) leads to an elevated number of theoretical plates. Conversely, a higher methanol concentration (X1) negatively affects the system by decreasing the theoretical plate count. Moreover, the interaction effects between factors demonstrated that the combined influence of methanol concentration and flow rate (X1X3), as well as pH and flow rate (X2X3), work together synergistically to improve the number of theoretical plates. However, the interaction involving methanol and flow rate (X1X3) also demonstrated a negative impact on the theoretical plates, suggesting a complex relationship among these factors.

$$\text{Theoretical plates (Y2)} = 14680 - 7.96X1 + 394.72X2 + 21.05X3 - 242.32X1X2 + 35.62X1X3 + 18.54X2X3 - 143.87X1^2 + 376.802X2^2 + 65.83X3^2 \quad (2)$$

Raising both the pH and flow rate leads to an increase in theoretical plates. Each factor was found to significantly influence the theoretical plates with a strong quadratic effect. This relationship is clearly demonstrated by the contour plot and the three-dimensional response surface graph.

To achieve optimal chromatographic performance, specific constraints were established for the factors and their corresponding responses. The finalized chromatographic parameters included a methanol composition (X1) set at 62%, a pH level (X2) adjusted to 1.7 and a flow rate (X3) maintained at 1 mL/min, collectively yielding a maximum desirability score of 1.00. Under these optimized conditions, the chromatogram of posaconazole is illustrated in Fig. 2. Experimental results from a checkpoint batch were compared against values predicted by the software to evaluate accuracy. The strength of the model was validated by conducting an analysis of variance (ANOVA) with the help of Design Expert software. In the

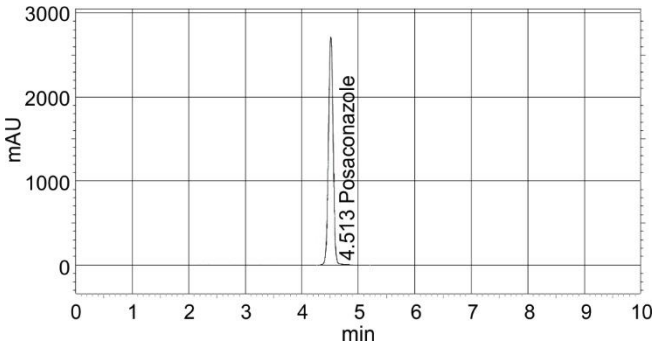

Fig. 2. Chromatogram of posaconazole in optimized chromatographic condition

subsequent point prediction step, the actual retention time observed was 4.393 min, which closely aligned with the predicted retention time of 4.513 min. Similarly, theoretical plate counts showed good agreement, with experimental and predicted values being 14,455 and 14,260, respectively, both within a 95% confidence interval. The calculated prediction errors fell within acceptable limits, supporting the model's strong predictive reliability and precision. Consequently, these findings validate the employed statistical design approach for chromatographic condition optimization, confirming its effectiveness and accuracy in predicting chromatographic behaviour. The statistical model showed strong reliability for both retention time and theoretical plates, with high R^2 values (0.993 and 0.987) and significant *p*-values (0.0001). Predicted R^2 values closely matched observed R^2 means, indicating accurate model fitting. Adequate precision values (16.15 and 32.27) confirmed the robustness and predictive efficiency of model [13].

Validated analytical method: The HPLC technique established for the analysis of posaconazole in the liposomal formulation was validated following the criteria outlined in the ICH Q2 (R1) guidelines [19].

System suitability: Prior to commencing the analysis of the samples, it is crucial to conduct system suitability testing to verify that the chromatographic apparatus is operating properly and consistently. To evaluate the performance of the system, six repeated measurements of posaconazole samples, each prepared at 1000 ppm, were carried out. The quantification of posaconazole was performed using a newly established HPLC technique. The peak area (31729871 ± 649.49) showed a very low %CV of 0.08, indicating consistent detector response. Retention time (4.393 ± 0.004 min) and tailing factor (1.043 ± 0.007) exhibited excellent reproducibility with %CVs of 0.03 and 0.48, respectively. The number of the theoretical plates (14964.17 ± 26.00) reflected high column efficiency (%CV 0.12), confirming the method's reliability for routine quality control analysis [20].

Selectivity: Fig. 3 shows the representative chromatograms of both the mobile phase and a 1000 ppm posaconazole solution. The analysis revealed that posaconazole exhibited a retention time of approximately 4.393 min.

Linearity: The standard curve of posaconazole was developed using HPLC at a wavelength of 262 nm, demonstrating excellent linearity with a regression coefficient (r^2) of 0.9996. The concentration range tested spanned from 800 to 1200 ppm.

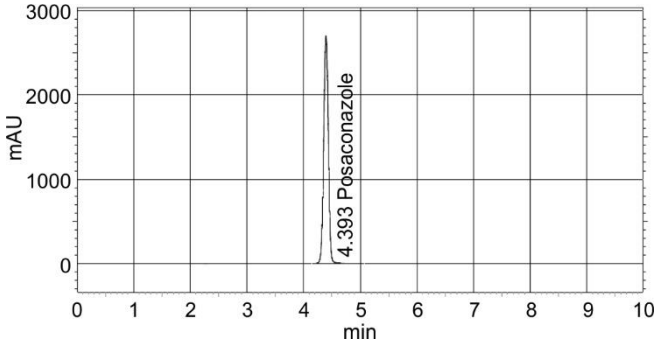

Fig. 3. Chromatogram of posaconazole in prepared liposomal formulation

Fig. 4 illustrates the standard curve generated under optimized chromatographic parameters, with the regression equation expressed as y = 26750x - 272211, confirming the precision and reliability of method for quantifying posaconazole within this range.

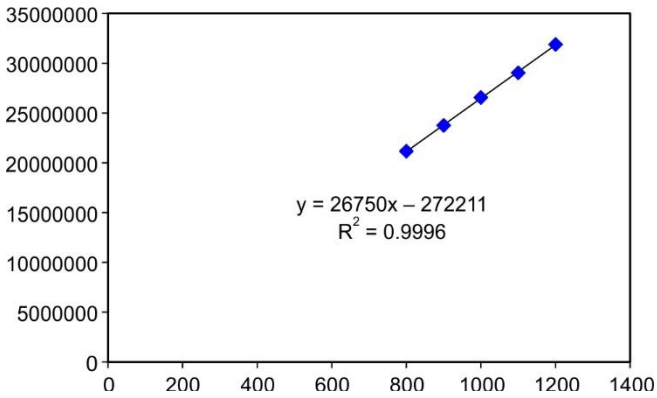

Fig. 4. Standard curve of posaconazole in optimized chromatographic conditions

Accuracy: The drug recovery percentages were observed to be 98.1 ± 0.12%, 99.8 ± 0.04% and 98.8 ± 0.05% at concentration levels of 80%, 100% and 120%, respectively. The accuracy assessment of the liposomal formulations revealed only slight differences compared to the reference standard. These minor discrepancies might result from the particular chemicals and solvents employed during the liposome preparation process. The percentage relative standard deviation (%RSD) values were recorded as 0.14, 0.13 and 0.16 for the respective concentrations, all of which fall within acceptable limits set for the evaluation. These findings indicate that the formulations maintained consistent accuracy and precision across the tested concentration range, adhering to the established criteria for method validation.

Precision: In line with the guidelines provided by ICH, the concentration of posaconazole was determined by conducting an assay at multiple time points within a single day as well as over the course of three consecutive days. This approach was undertaken to thoroughly assess the precision of the analytical method. To evaluate this, six replicate measurements of a 1000 ppm posaconazole solution were performed. The results of the amount of drug for intra-day and inter-day precision studies found during the intra-day analysis ranged from 991.2 ± 3.77 µg/mL in morning to 993.6 ± 2.47 µg/mL in evening, while inter-day values varied from 992.5 ± 4.74 µg/mL on Day 1 to 998.5 ± 2.81 µg/mL on Day 2. The percent amount found ranged from 99.12% to 99.85%, with all relative standard deviations (RSDs) under 0.40%, demonstrating high analytical precision.

Robustness: Alterations in the mobile phase composition (methanol:buffer 70:30 v/v and 60:40 v/v, pH 1.7) resulted in stable peak areas (mean ± SD: 31,724,838 ± 319 and 32,829, 467 ± 684) with extremely low %RSD values (0.041% and 0.027%), indicating excellent reproducibility [21]. Theoretical plate numbers remained high (~14,363-14,549) and the tailing factor was close to unity (1.06-1.09), demonstrating good column efficiency and symmetrical peak shapes. When the flow rate was changed from 1.0 to 1.5 mL/min, only slight differences in peak area and theoretical plates were observed, with all %RSDs still within acceptable limits (≤ 0.22%) and the tailing factor remained unchanged at 1.06, reflecting consistent peak integrity. Adjustments in pH (from 1 to 2) showed acceptable variation in peak area and theoretical plates, while the tailing factor stayed consistent at 1.05, confirming the method's tolerance to slight pH fluctuations. Variations in the injection volume (5 µL *vs.* 10 µL) affected peak area as expected but maintained low %RSD values (≤ 0.14%) and good system suitability with theoretical plates around 13,764-14,273 and tailing factors at 1.05. Oven temperature changes (40 ºC and 50 ºC) also did not significantly affect method performance, as peak area, theoretical plates and tailing factors remained within acceptable variability limits. Overall, all parameters demonstrated %RSDs well below 2%, confirming that the method is highly robust and reliable for routine analytical applications under varied operational conditions (Table-2).

The robustness study outcomes indicate that the analytical method is highly stable and resilient to small, deliberate changes in the chromatographic conditions. Consistently low %RSD values across all parameters, peak area, theoretical plates and tailing factor, highlight the method's precision and reliability. The minimal variation in peak area despite changes in mobile phase composition, flow rate, pH, injection volume and oven temperature suggest that the method maintains its quantitative accuracy under routine fluctuations [22]. Theoretical plate counts remained high across all conditions, reflecting excellent column efficiency, while tailing factors consistently hovered around 1.05-1.09, confirming good peak symmetry and minimal distortion. Importantly, none of the tested variations led to a loss in resolution or sensitivity, indicating that the method is specific and robust enough to be used across laboratory settings and analyst conditions. Such robustness ensures that the method can withstand minor operational variations without compromising data quality. It is well-suited for quality control and routine analytical workflows in pharmaceutical and liposomal formulation studies.

Analysis of liposomal formulation: The analytical method developed for the liposomal formulation exhibited excellent linearity across the concentration range of 800-1200 µg/mL, with a high regression coefficient (R^2 = 0.9994), a slope of 26953 and an intercept of 564590. Precision was confirmed through low intra-day (0.12% RSD) and inter-day (0.36-0.39% RSD) variations. The method demonstrated high accuracy with recovery values ranging from 98.4% to 99.4%. Ruggedness was established by consistent results from two

TABLE-2
ROBUSTNESS OF THE DEVELOPED HPLC METHOD FOR PCZ DETERMINATION

Conditions	Levels	Peak area		Theoretical plates		Tailing factor	
		Mean ± SD	%RSD	Mean ± SD	%RSD	Mean ± SD	%RSD
Mobile phase composition (methanol: buffer (70:30 v/v pH 1.7)	70:30	31724838 ± 319	0.041	14549 ± 62.73	0.14	1.09 ± 0.004	0.35
	60:40	32829467 ± 684	0.027	14363 ± 49.38	0.16	1.06 ± 0.004	0.38
Flow rate (mL/min)	1.0	3223343 ± 1181	0.05	14058 ± 83.73	0.22	1.06 ± 0.02	0.34
	1.5	3310921 ± 1169	0.05	14188 ± 95.68	0.12	1.06 ± 0.05	0.29
pH	1	3106382 ± 1294	0.11	14747 ± 67.45	0.23	1.05 ± 0.06	0.36
	2	3257429 ± 3847	0.13	13388 ± 28.20	0.16	1.05 ± 0.03	0.64
Injection volume (µL)	05	3318537 ± 4658	0.13	14273 ± 84.51	0.16	1.05 ± 0.06	0.87
	10	3219293 ± 9373	0.14	13764 ± 76.15	0.18	1.05 ± 0.04	0.78
Oven temperature (ºC)	40	3123847 ± 3748	0.16	13848 ± 48.19	0.16	1.06 ± 0.07	0.48
	50	31283484 ± 8474	0.19	14748 ± 94.09	0.18	1.05 ± 0.04	0.42

different analysts, yielding RSDs of 1.42% and 1.38%, respectively. Thus, the method was found to be robust and specific and the assay of the liposomal formulation yielded a value of 97.5 ± 0.41%. The summarized details of the method can be found in Table-3.

TABLE-3
SUMMARY OF THE PROPOSED RP-HPLC METHOD'S REGRESSION, VALIDATION AND IN-HOSE LIPOSOMAL FORMULATION ASSAY PARAMETERS

Sl. No.	Parameters	Results
1	Regression coefficient	0.9994
2	Slope	26953
3	Intercept	564590
4	Linearity range (µg/mL)	800-1200
5	Precision (n = 6, RSD, %)	
Intra-day		0.12
Inter-day		0.36-0.39
6	Accuracy	98.4-99.4%
7	Ruggedness	
Analysts I (n = 3, RSD, %)		1.42
Analysts II (n = 3, RSD, %)		1.38
8	Robustness	Robust
9	Specificity	Specific
10	Liposomal formulation assay (%)	97.5 ± 0.41

These validation results confirm that the method is reliable, reproducible and suitable for quantitative analysis of the liposomal formulation. The excellent linearity and high correlation coefficient ensure the method's applicability across a wide concentration range. The low RSD values for precision and ruggedness demonstrate consistent performance under both repeatability and intermediate precision conditions. High accuracy reflects minimal systematic errors and ensures confidence in the assay results. The robustness and specificity further support the method's reliability even under slight variations in the experimental conditions or in the presence of formulation excipients. The liposomal formulation assay result of 97.5% ± 0.41% indicates a high degree of content uniformmity, making this method appropriate for routine quality control analysis.

Force degradation: Forced degradation studies were conducted to evaluate the chemical stability of posaconazole under various stress conditions, including acidic and basic environments, oxidative agents, elevated temperatures, and light exposure. Samples were analyzed using HPLC to quantify the remaining posaconazole based on its chromatographic peak area. The extent of degradation was determined by calculating the percentage loss relative to a control, providing insight into the drug's stability profile under different environmental stresses. The results are presented in Table-4.

Acid hydrolysis: Posaconazole was subjected to hydrolytic degradation in acidic media using different concentrations and volumes of HCl. The results indicated that degradation increased with both the concentration and volume of HCl. At 0.1 M HCl, the degradation was minimal (0.23% with 5 mL and 0.39% with 10 mL), while at 1.0 M HCl, the degradation

TABLE-4
FORCED DEGRADATION STUDY OF POSACONAZOLE (1000 ppm)

Stress condition	Treatment	Concentration	Volume	Area	Degradation (%)
Untreated sample	–	–	–	26,552,517.83	–
Acid hydrolysis (1 h)	0.1 M HCl	Mild	5 mL	26,492,046	0.23
	0.1 M HCl	Mild	10 mL	26,448,264	0.39
	1.0 M HCl	Strong	5 mL	26,249,264	1.14
	1.0 M HCl	Strong	10 mL	25,895,263	2.48
Base hydrolysis (1 h)	0.1 M NaOH	Mild	5 mL	26,534,546	0.07
	0.1 M NaOH	Mild	10 mL	26,502,546	0.19
	1.0 M NaOH	Strong	5 mL	26,305,264	0.93
	1.0 M NaOH	Strong	10 mL	25,999,263	2.08
Oxidative (1 h)	H_2O_2	10%	5 mL	26,548,656	0.015
	H_2O_2	10%	10 mL	26,546,656	0.022
Thermal (70 ºC, 24 h)	Dry heat	–	–	26,550,517	0.008
Photolytic (24 h light)	UV 245 nm exposure	–	–	26,541,173	0.04

significantly increased to 1.14% and 2.48% for 5 mL and 10 mL volumes, respectively.

Base hydrolysis: The basic degradation was performed using NaOH at two concentrations (0.1 M and 1.0 M) and two volumes (5 mL and 10 mL). Similar to acid hydrolysis, an increase in concentration and volume led to a greater extent of degradation. At 0.1 M NaOH, the degradation was negligible (0.07% and 0.19%), while at 1.0 M NaOH, it increased to 0.93% and 2.08% for 5 mL and 10 mL, respectively.

Oxidative hydrolysis: Oxidative stress was induced using 10% H_2O_2 at two volumes (5 mL and 10 mL). The degradation observed was minimal, with 0.015% degradation in 5 mL condition and only 0.022% with 10 mL indicating that posaconazole is highly stable under oxidative conditions for short durations.

Thermal degradation: Posaconazole was subjected to dry heat at 70 ºC for 24 h. The resulting degradation was negligible, with only 0.008% observed, suggesting excellent thermal stability.

Photolytic degradation: Exposure to UV light for 24 h resulted in a very slight degradation of posaconazole, with a degradation of 0.04%, indicating that the drug is relatively photostable under these test conditions. Overall, the results demonstrate that posaconazole is highly stable under oxidative, thermal and photolytic conditions, while showing slight to moderate degradation in acidic and basic environments, particularly under higher concentrations and longer exposures.

Posaconazole exhibited robust chemical stability under oxidative, thermal and photolytic conditions, as evidenced by minimal degradation values ranging from 0.008% to 0.04%. However, the drug was moderately susceptible to hydrolytic degradation under strongly acidic and basic environments. Degradation increased with both the strength and volume of the acid or base, peaking at 2.48% in 1.0 M HCl and 2.08% in 1.0 M NaOH (10 mL each). These findings suggest that while posaconazole is generally stable, formulations should avoid prolonged exposure to extreme pH to maintain drug integrity. The overall degradation under all stress conditions remained below 5%, supporting its suitability for formulation development.

The application of response surface methodology enabled systematic optimization of the RP-HPLC method for posaconazole in the liposomal formulations. By analyzing the interactions between formulation variables and quality attributes, the study achieved efficient chromatographic performance with retention times between 3.92-4.86 min and theoretical plates up to 14,954. The UV absorbance peak at 262 nm further confirmed posaconazole detection, validating the method's precision and robustness across the experimental trials.

The study evaluated the influence of critical material attributes on key critical quality attributes (CQAs), with a focus on retention time (Y1) and theoretical plates (Y2). A polynomial model showed that methanol content (X1) had a negative impact on retention time, whereas pH (X2) and flow rate (X3) had positive effects. These findings indicate that retention time increases with higher pH and flow rate within optimal limits, and with lower methanol content. The model accurately predicted retention time using coded factor levels and identified significant main effects and interactions.

For theoretical plates, pH and flow rate positively influenced the system efficiency, while methanol content had a negative effect. Interactions such as X2X3 and X1X3 revealed complex, synergistic relationships, highlighting the multifactorial nature of chromatographic optimization. The optimal conditions, 62% methanol, pH 1.7, and 1 mL/min flow rate, achieved a desirability score of 1.00. The experimental values closely matched predictions for Y1 and Y2, with minimal errors and high R^2 values (0.993 and 0.987), confirming the model's robustness and predictive power.

System suitability confirmed the proposed method readiness for accurate analysis. Six replicate injections of 1000 ppm posaconazole yielded consistent peak areas (31729871 ± 649.49, %CV = 0.08), highly reproducible retention times (4.393 ± 0.004 min, %CV = 0.03) and tailing factors (1.043 ± 0.007, %CV = 0.48). High theoretical plate counts (14964.17 ± 26.00, %CV = 0.12) indicated excellent column efficiency and system stability. The method exhibited high selectivity, with posaconazole eluting sharply at 4.393 min, free of interference from the liposomal matrix. Chromatograms of the mobile phase showed no interfering peaks, confirming specificity. Linearity was excellent across 800-1200 ppm (R^2 = 0.9996), following the equation y = 26750x – 272211, indicating the strong proportionality between concentration and response.

Recovery studies at 80%, 100%, and 120% yielded mean recoveries of 98.1%, 99.8% and 98.8%, with low %RSDs (0.14-0.16), confirming method accuracy. Minor deviations were attributed to excipients or solvents in the matrix. Intra-day (0.18-0.19%) and inter-day (0.30-0.36%) %RSDs demonstrated high precision and reproducibility. Morning and evening injections showed minimal variability, affirming short-term stability, while consistent results across three days confirmed long-term reliability.

Robustness results showed the method remained precise and consistent under deliberate variations in chromatographic conditions (mobile phase ratio, pH, flow rate, injection volume, oven temperature), with all %RSDs below 2%. Column efficiency and peak symmetry remained unaffected, supporting the method's reliability for routine pharmaceutical analysis. The final method demonstrated excellent linearity (R^2 = 0.9994), high precision, accuracy (98.4-99.4%) and ruggedness across analysts. Assay results (97.5 ± 0.41%) confirmed uniformity. Posaconazole remained stable under oxidative, thermal and photolytic stress (< 0.05% degradation), but showed moderate degradation under acidic and basic conditions, particularly at higher strengths, 2.48% in 1.0 M HCl and 2.08% in 1.0 M NaOH. These findings emphasize the sensitivity of drug to extreme pH and the importance of pH control in formulation and storage. Overall degradation remained below 5%, confirming the chemical robustness of posaconazole for pharmaceutical use.

Conclusion

This study focussed on the development, optimization and validation of a robust reverse-phase high-performance liquid chromatography (RP-HPLC) method for quantifying posaconazole in the liposomal formulations. Using response surface methodology (RSM), key variables, methanol concentration,

pH and flow rate, were optimized through polynomial equations and contour plots. The optimized method achieved a retention time of 4.393 min, with excellent system suitability, including theoretical plates over 14,000 and tailing factors near unity. Validation followed ICH Q2(R1) guidelines, demonstrating linearity across 800-1200 µg/mL (R^2 = 0.9994), high precision (intra-day RSD: 0.12%; inter-day RSD: 0.36-0.39%) and accuracy ranging from 98.4% to 99.4%. Ruggedness was confirmed with analyst-to-analyst RSDs below 1.5% and robustness testing under varied conditions yielded RSDs under 2%, confirming method reliability. The assay of the liposomal formulation showed 97.5 ± 0.41% drug content, supporting routine applicability. Forced degradation studies demonstrated that posaconazole remains stable under oxidative, thermal and photolytic conditions, while moderate degradation was observed under acidic and basic hydrolysis, particularly at higher concentrations. Overall the degradation levels remained below 5%, indicating satisfactory chemical stability. In conclusion, the developed method is precise, robust and stability indicating, making it highly suitable for routine quantitative analysis and quality control of liposomal posaconazole formulations

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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