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Analytical Quality by Design Assisted Optimization of RP-HPLC Method for the Estimation of Palovarotene Drug Substance and Drug Product by Box–Behnken Design

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A QbD approach, with its emphasis on risk assessment and management, may result in the establishment of a more robust or rugged system. Critical quality attributes (CQAs) and the analytical target profile were evaluated by closely examining the detailed process for analytical QbD-based optimization parameters. RP- HPLC is considered more useful than normal phase HPLC method since it is more versatile in separating a wider range of compounds, due to its non-polar stationary phase and polar mobile phase, allowing for better control over Rt and mobile phase composition, with improved reproducibility and accuracy compared to normal phase HPLC. The current study outlines the invention and validation of the straightforward, quick, sensitive, and affordable RP-HPLC approach for investigating palovarotene in tablet formulations. Three essential elements of the RP-HPLC approach buffer pH, flow rate and ratio of MP were used in the Box-Behnken design factor screening investigations. The DOE trial version 12.0 was used to optimize the chromatographic settings. With water Platisil C18-EP (4.6 x 250 mm, 5μm) column and comprising of a mobile phase KH2PO4 (pH 3.5): ACN (50:50 ml) v/v, a 1.0 ml/min flow rate and UV range at 261 nm, the best chromatographic separation was accomplished. The interrelationships between MP, pH, and flow rate at 3 distinct levels are described by the Box–Behnken experimental design. RSM plots and statistical data were used to evaluate the retention duration and theoretical plate responses. The current RP-HPLC method for palovarotene was in compliance with the suggested ICH recommendations. The technique can also be utilized for quality control and laboratories evaluation aimed at the assessment of Palovarotene in the drug material and capsule formulation.

Keywords: Analytical Quality by Design; Box Behnken Design; Fibrodysplasia Ossificans Progressive; Factors and Responses; Palovarotene.

Palovarotene (Sohonos, Ipsen, Paris, France) was approved by the USFDA on August 16, 2023, as a therapeutic option for fibrodysplasia ossificans progressive (FOP), after being approved in Canada in 2022. [1] By binding to the retinoic acid receptor ã (RARã), palovarotene suppresses

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BMP signaling; hence, chondrogenesis and, ultimately, HO is hindered. [2] A bone morphogenetic protein (BMP) point mutation in the type I receptor is the cause of FOP, an incredibly rare autosomal dominant disorder. [3] Palovarotene is a selective agonist of the transcriptional repressor retinoic acid receptor gamma (RARã), which is expressed in chondrocytes and chondrogenic cells. [1] Palovarotene suppresses the SMAD1/5/8 signaling pathway by reducing BMP signaling by its binding to RARã. [2]

Palovarotene reduces muscle tissue damage by interfering with these pathways, which decreases chondrogenesis and permits normal muscle tissue repair. [3]

No analytical techniques, including HPLC and UV, have been documented for the assessment of palovarotene in capsule formulation and API, and no studies on quality by design approach has been reported, according to literature reviews. Finding failure models and developing a reliable approach with adjustable design space for the duration of product life cycle management has been the main goal of AQbD [4].

The present research aims to use analytical QbD principles to establish and optimize the RP-HPLC approach for estimating the capsule formulation of palovarotene. To achieve high resilience and improve method performance, the QbD concept has been widely used in the assessment of analytical parameters. [5,6] QbD was

Table 1. Optimization of parameters using BBD Design for Palovarotene

Run	F1 Buffer pH	F2 Mobile Phase	F3 Flow Rate	R1 RT	R2 Plate Count
1	3.50	50.00	1.00	3.7	19489
2	3.50	50.00	0.80	3.9	19589
3	4.50	30.00	0.90	2.1	12532
4	3.50	40.00	0.90	3.0	16452
5	2.50	40.00	0.80	2.0	13267
6	3.50	40.00	1.00	3.4	17234
7	3.50	40.00	1.00	3.4	17234
8	4.50	40.00	0.80	3.1	15765
9	3.50	40.00	1.00	3.4	17234
10	3.50	30.00	1.00	3.6	18678
11	3.50	40.00	0.90	3.4	17234
12	2.50	40.00	1.00	4.0	19699
13	4.50	40.00	1.00	2.9	14798
14	4.50	50.00	0.90	2.7	13987
15	3.50	30.00	0.80	4.1	19733
16	2.50	30.00	0.90	4.2	19853
17	2.50	50.00	0.90	4.4	19900

Table 2. Selection of Levels for BBD Design for Palovarotene

Independent Variables	Levels		
	-1	0	+1
buffer pH	2.5	3.5	4.5
Mobile Phase	30	40	50
Flow Rate	0.8	0.9	1

Table 3. Fit Summary Response of Retention Time for Palovarotene

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	< 0.0001		0.9071	0.8520	
2FI	0.9786		0.8815	0.6607	
Quadratic	< 0.0001		0.9943	0.9602	Suggested
Cubic			1.0000		Aliased

discovered on the knowledge and application of (ICH Q8), (ICH Q9) and guidelines (ICH Q10). [7,8]

MATERIALS AND METHODS

Chemicals

The Palovarotene API was gifted by MSN Labs Pvt., Ltd, Hyderabad, India. The marketed capsule formulation of Sohonos (Palovarotene 5 mg) was used for assay. Potassium dihydrogen phosphate & acetonitrile HPLC grade were purchased from Sigma Aldrich, Mumbai, India.

Instruments and Equipment

The technique was developed using a HPLC (Shimadzu) equipped system with a quaternary pump, a sonicator, sample, detector (PDA) (software) LC solution. Weighing machine (Shimadzu).

Stock solution (Standard)

Palovarotene 25 mg was measured in 25 ml VF and mixed with CH_3OH to prepare stock solution (1000ìg/ml). By serially diluting this stock solution with methanol, several std solutions (10–60ìg/ml) were enumerated. A nylon filter (0.45 ì) was used to filter the produced solution, and chromatographic analysis was performed to enumerate the calibration plot.

Sample solution Preparation

Desired amount 25 mg of palovarotene was measured and taken in a 25 ml VF, after adding 15 ml of methanol to the flask was shaken, sonicate it for 15 minutes. After shaking the flask, diluent was added to make it up to par. A nylon filter (0.45 ì) was used to filter the aforementioned solution. The sample solution was brought up to the mark with desired diluent solution to prepare a sample containing 20 ìg/ml of Palovarotene and assessed.

Table 4. (R2) Plate Count of Palovarotene ANOVA for Quadratic model

Sources	Sum of square	df	Mean square	F value	P value Prob>F	
Mean VsTotal	5.627E+009	1	5.627E+009			Suggested
Linear vsMean	7.254E+006	3	2.418E+006	1.98	0.1662	Suggested
2F Vs linear	1.668E+006	3	5.559E+005	0.39	0.7614	
Quadratic	1.236E+007	3	4.122E+006	15.93	0.0016	
Cubic VsQuadra	1.762E+006	3	5.874E+005	47.76	0.0014	Aliased
Residual	49203.20	4	12300.80			
Total	5.650E+009	17	3.323E+008			

Table 5. Response 1 Retention Time of Palovarotene ANOVA for Quadratic model

Sources	Sum of square	df	Mean square	F value	P value Prob>F	
Mean VsTotal	193.13	1	193.13			Suggested
Linear vsMean	2.02	3	0.63	1.60	0.2382	Suggested
2F Vs linear	1.27	3	0.42	1.01	0.4289	
Quadratic	1.39	3	0.46	1.15	0.3930	
Cubic VsQuadra	2.69	3	0.90	27.99	0.0038	Aliased
Residual	0.13	4	0.032			
Total	200.63	17	11.80			

Table 6. System Suitability studies for Palovarotene

S. No	Name	RT (min)	Area (µV sec)	Height (µV)	USP tailing	USP plate count
1	Palovarotene(Standard)	2.970	1843335	23449	1.15	5804
2	Palovarotene(Sample)	2.978	1716576	27492	1.14	5411

Method Development by QBD Approach

Analytical target profile

The construction of the ATP, is critical to determining the variables required to guarantee the quality attributes and goal of the analytical process. The target profile for effective method development includes an analyst, sample, and a variety of analytical techniques.[9]

Critical Quality Attributes (CQA) Determination

CQAs are selected for AQbD analysis depends on the methods utilized to develop a method (RP-HPLC) and (e.g. assay and drug delivery assessment). The CQAs for the assay analysis procedure include tailing factor (Tf), plate count (N), resolution, Rt, and % recovery.[10,11]

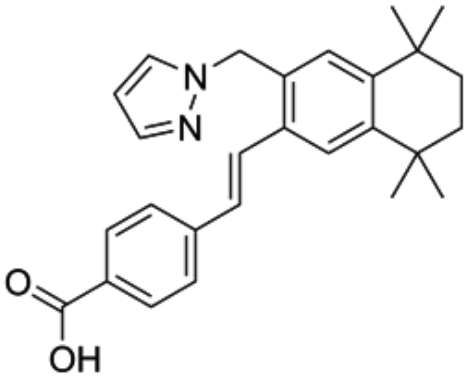

Fig. 1. Structure of Palovarotene

Risk assessment and Identification of Critical Method Parameters (CMP) of the method

CMPs refer to analytical procedure sensitivity. The relationship between CMPs and CQAs is straightforward. CMPs are classified using a variety of analytical procedures such as GC, HPLC, and HPTLC. Column temperature, flow rate, injection volume, mobile phase pH, and organic modifier % are the various CMPs for developing RP-HPLC methods. Risk assessment evaluates the likelihood of subsequent failure and interactions with CMPs.[11,12]

Design of Experiment (DOE)

RSM model was chosen for DOE selection in contrast to factorial design. Because Box-Behnken provided a better interaction with a 3-factor and 3-level design with fewer runs than the BBD, it was selected for additional research. Three levels of Box–Behnken response surface design was used to optimize the method performance based on factor screening studies: low (“1), medium (0), and high (+1). CQAs (Rt and Tf) are chosen as dependent variables in DOE, while specific CMPs factors (pH of buffer, flow rate, and percentage organic modifier) are chosen as input variables (Table 1).[12,13]

Method development and Validation

Accuracy

The degree of agreement between the actual value and the discovered value is referred to as accuracy, also known as trueness. The %

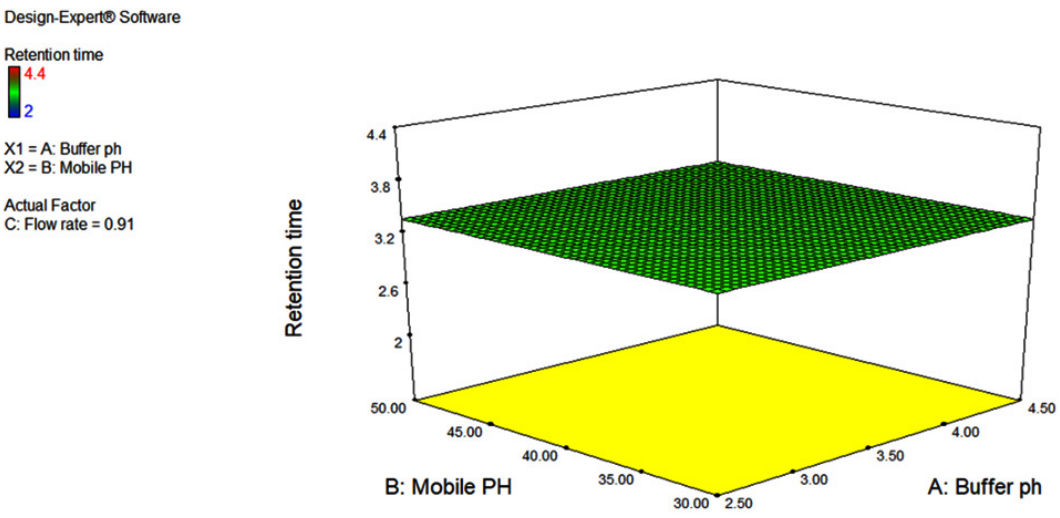

Fig. 2. 3D Surface Plot of Retention time for Palovarotene

recovery was estimated by adding three different quantities of recognized standards to previously analysed samples.

Precision

Precision is the degree to which a series of measurements conducted under predetermined conditions with repeated samples of a different homogenous substance agree closely. It is referred to as %RSD.

Intermediate precision refers to the diversity within laboratories caused by different analysts, instruments, days, and so on. This is commonly referred to as method precision. Six separate sample results taken on six different days were used in the evaluation.

Linearity

A technique's ability to analyse and deliver results proportional to the analyte concentration of the sample within a given range. To determine linearity, the regression equation of the calibration curve was used, which was derived from six linear standard concentrations.

Robustness

In terms of deliberate changes in method parameters, the method's validity was tested by varying the mobile phase composition and flow rate.

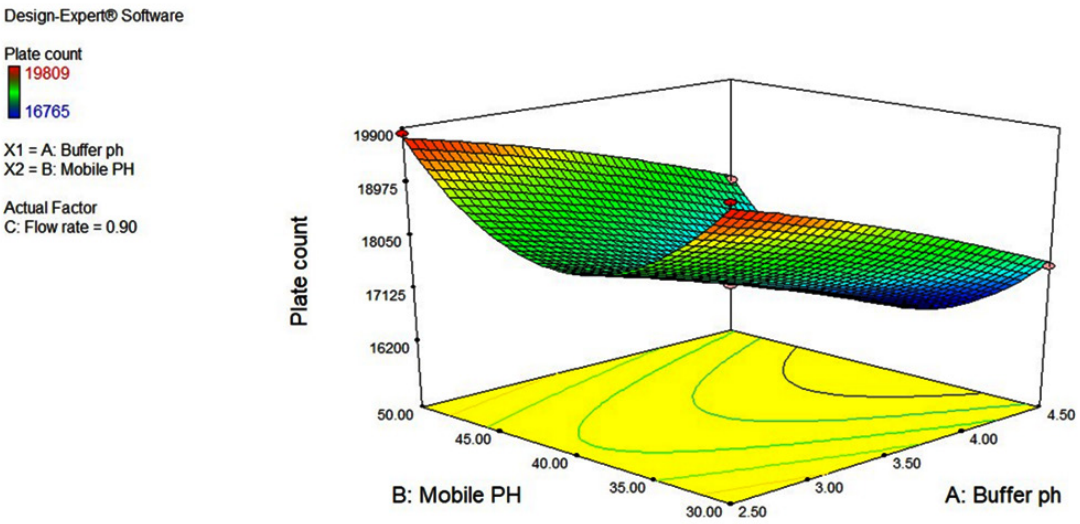

Fig. 3. 3D Surface Plot of Plate Count for Palovarotene

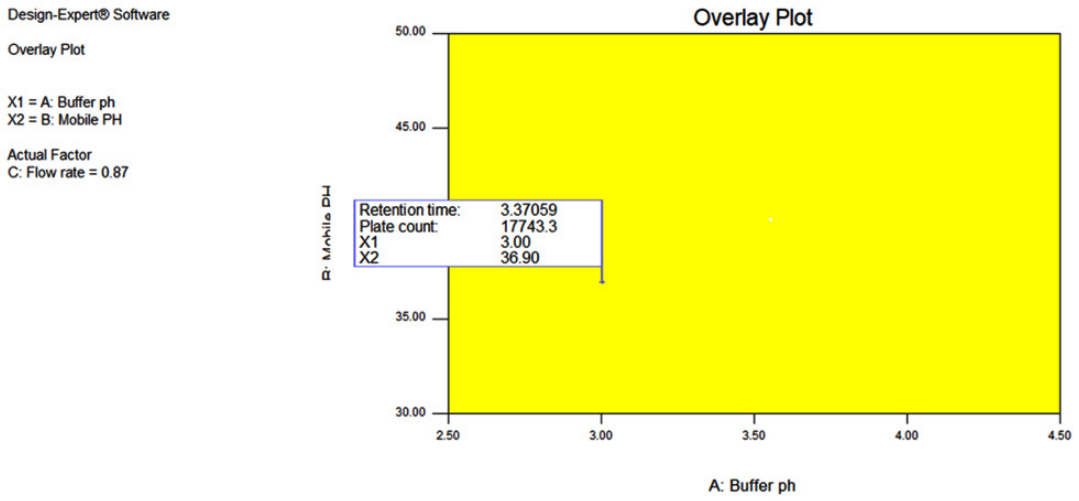

Fig. 4. Overlay plot for Palovarotene

LOD & LOQ

Detection and quantification limits were assessed for Palovarotene at 3:1 and 10:1 S/N ratio respectively, by analysing series of known concentration dilutions.

System suitability

System suitability testing was undertaken to ensure that the measurement system and analytical technique were appropriate for the proposed analysis. Six indistinguishable samples were tested.

RESULTS

A DOE listing 17 runs was generated utilizing Stat Ease software. The BBD screening mechanism was used to investigate the various interaction and linear models of the buffer pH flow rate and MP ratio, on the retention period and tailing factor (Table 2-6). [14,15]

HPLC Optimization by QbD approach

To optimize HPLC conditions, Rt, and theoretical plate (N), were chosen as ATPs. The BBD was utilized to refine the optimising

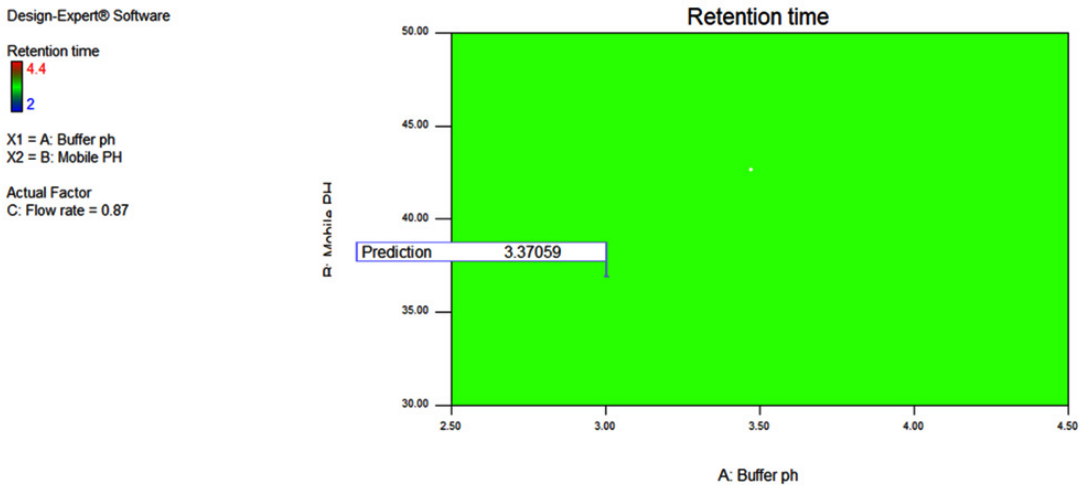

Fig. 5. Retention Time for Palovarotene

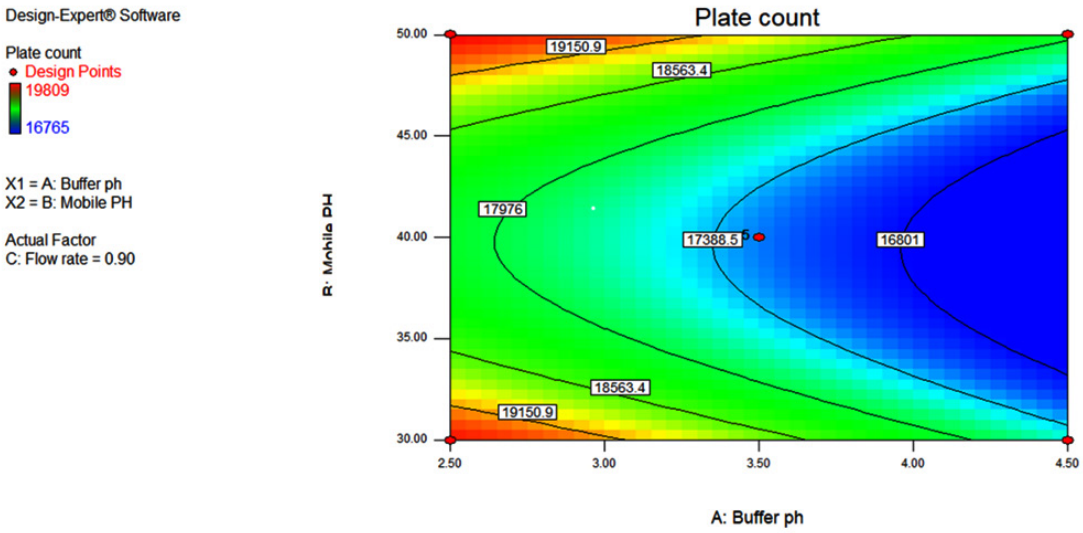

Fig. 6. Plate count factor for Palovarotene

numerous factors within the operational space. For the main impact of interaction, the quadratic model was used in the analysis. The optimized variables for the model are displayed in Table 2. For each CAA parameter, the model produces a quadratic equation. When the mobile phase ratio and pH decreases retention time will increase and also theoretical plate count increases. [9] The optimized method exhibited the mobile phase mixture comprising 50:50% v/v mix of KH_2PO_4:

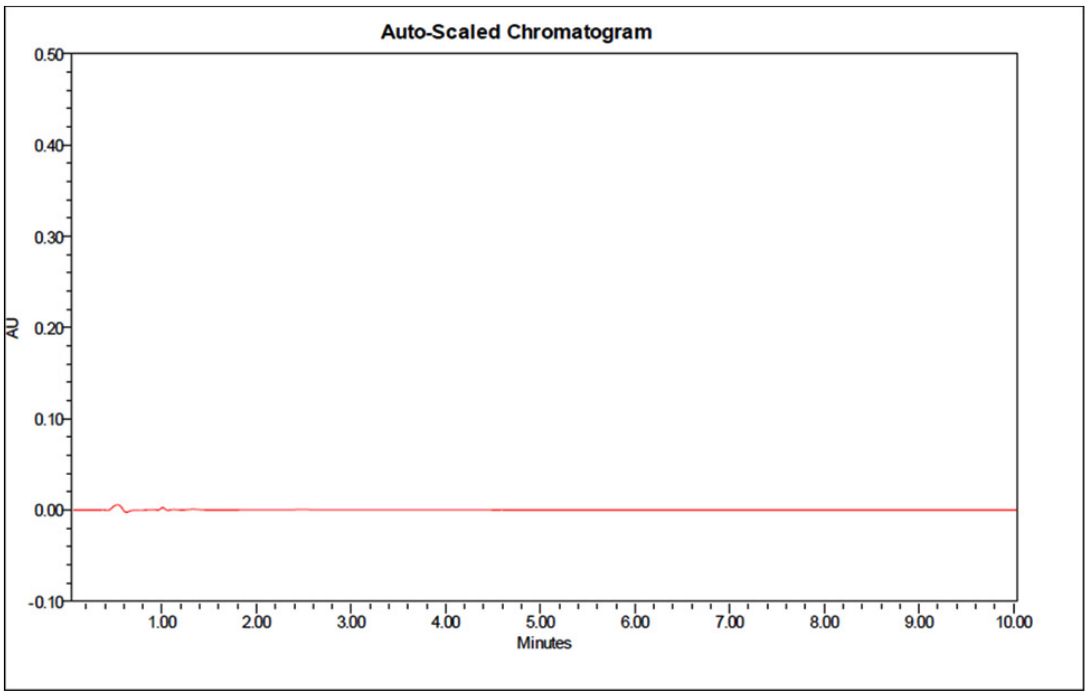

Fig. 7. Chromatogram of Blank

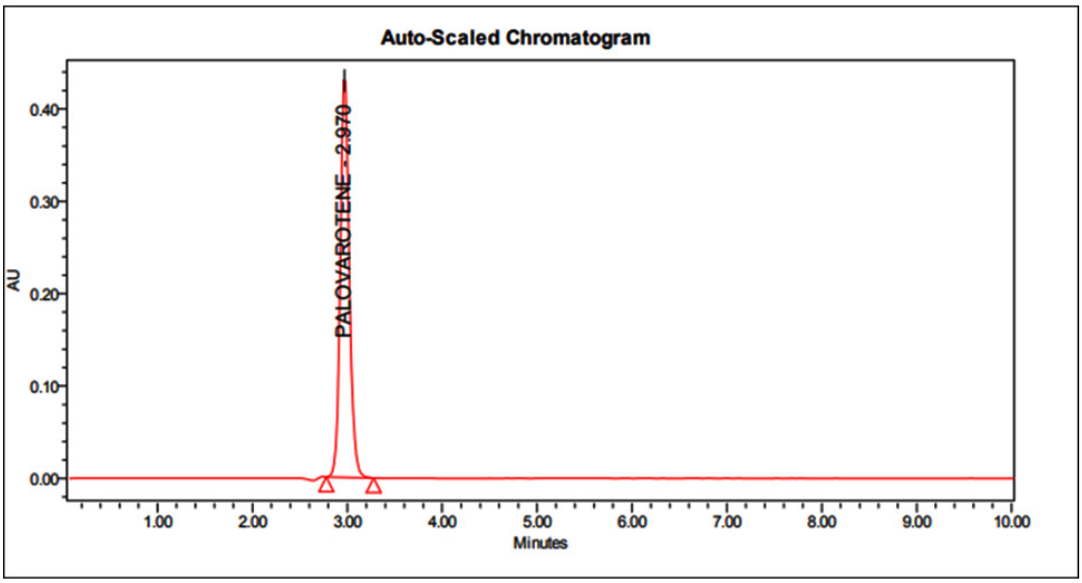

Fig. 8. Chromatogram for Standard

(pH 3.5) ACN and 1 ml/min flow rate achieved values close to 1.0 along with all the CAAs in the intended range (Figure 2-6). [16,17]

System suitability

The Overall, system suitability parameters of Palovarotene were within the range and are acceptable as per ICH guidelines and are presented in Table 6 & Figure 7 Chromatogram of Blank, Figure 8 Chromatogram of Standard and Figure 9 Chromatogram of Sample.

Method validation studies

Linearity

The technique's linearity was recognized by plotting a calibration curve for the individual

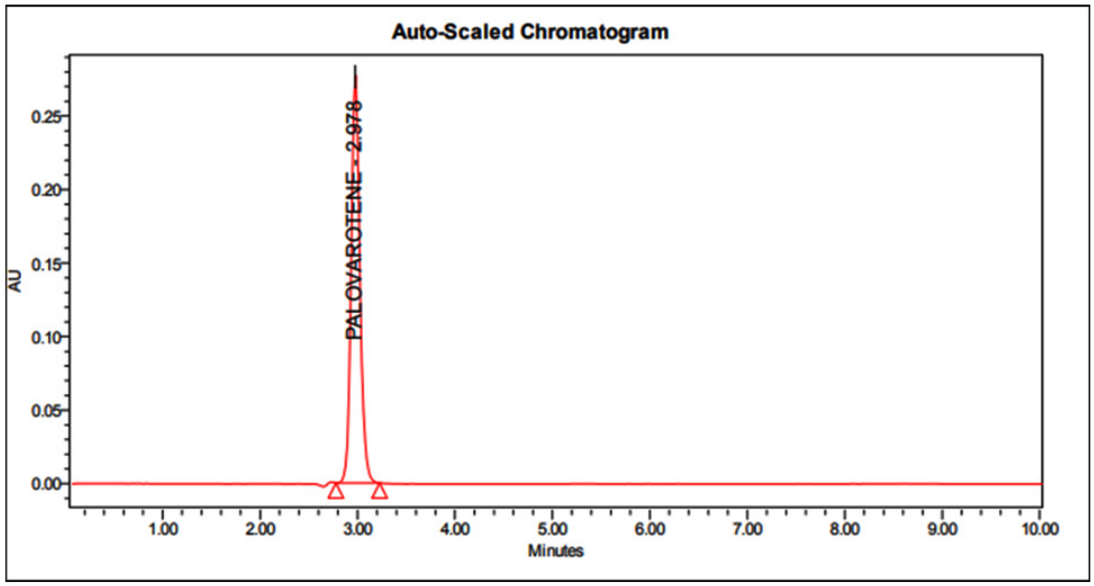

Fig. 9. Chromatogram for Sample

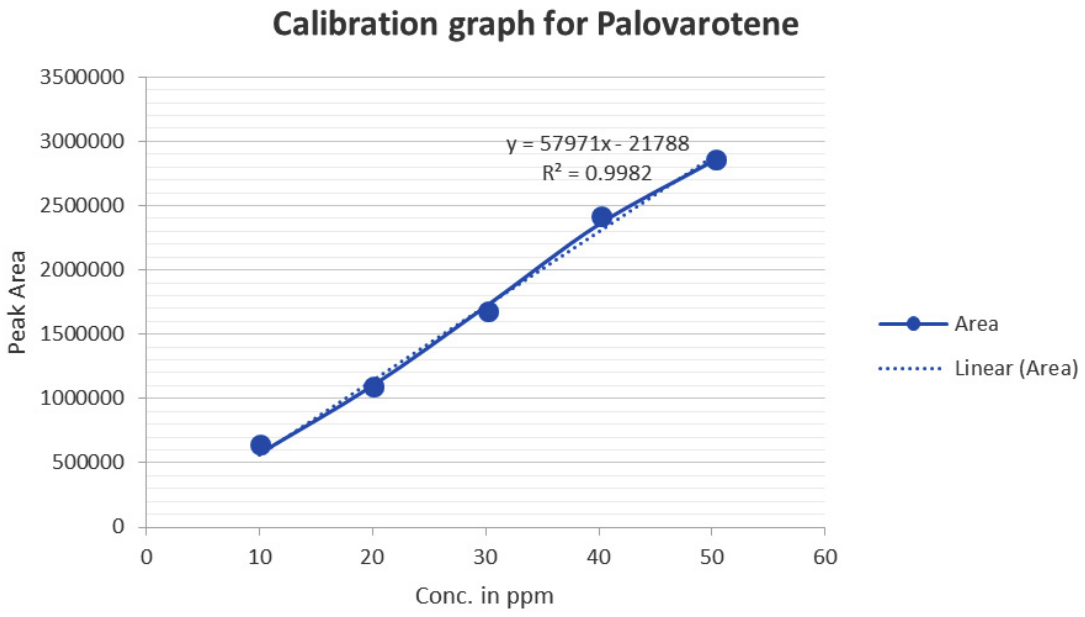

Fig. 10. Calibration graph for Palovarotene

Table 7. Area of different concentration of Palovarotene

S. No	Palovarotene Concentration (µg/ml)	Area
1	10	572192
2	20	1099896
3	30	1716576
4	40	2354708
5	50	2843335

drug's concentration level and their corresponding peak area. It was perceived on the concentration scale of 10- 50µg/ml of Palovarotene. The coefficient of determination, R^2 was noticed to be not more than 0.999% and therefore the technique is linear. The respective results are given in Table 7. The calibration curves of Palovarotene are shown in Figure 10.

Accuracy

Three concentrations levels 50%, 100% and 150% were considered for accuracy and the mean % recovery was 99.52 to 99.93 % for Palovarotene which was satisfactory and manifest the trueness of the technique. The results are as given in Table 8.

Table 8. Accuracy (recovery) data for Palovarotene

% Concentration (at specification Level)	Area*	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	858288	25	24.88	99.52	99.76
100%	1716576	50	49.92	99.84	
150%	2574864	75	74.95	99.93	

Table 9. Results of Precision for Palovarotene

Injection	Area	Injection	Area
Injection-1	1681068	Injection-1	1671068
Injection-2	1668199	Injection-2	1668199
Injection-3	1681077	Injection-3	1681077
Injection-4	1681077	Injection-4	1671077
Injection-5	1677489	Injection-5	1677489
Injection-6	1684877	Injection-6	1684877
Average	1678965	Average	1675631
Standard Deviation	5768.549	Standard Deviation	6563.446
%RSD	0.34	%RSD	0.39

Table 10. LOD & LOQ values of Palovarotene

Drug name	Baseline noise (µV)	Signal obtained (µV)	S/N ratio	Conc.
Palovarotene	93	276	2.96	0.3 µg/ml
		Limit Of Quantification (LOQ)		
Palovarotene	93	926	9.95	1.0 µg/ml

Table 11. Results for variation in flow for Palovarotene

S. No	Flow Rate (ml/min)	System Suitability Results USP Plate Count	USP Tailing
1	0.8	5405	1.13
2	1	5411	1.14
3	1.2	5415	1.09

Precision

Intermediate precision and Repeatability were considered and the % RSD was analysed to be within the acceptable range. The results are as given in Table 9.

Table 12. Results for variation in mobile phase composition for Palovarotene

S. No	Change in Organic Composition in the Mobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10 % less (45 ml)	5405	1.13
2	*Actual (50 ml)	5411	1.14
3	10 % more (55 ml)	5415	1.09

LOD & LOQ

The observed values of LOD were 0.3ìg/ml and the LOQ were 1.0ìg/ml respectively for the Palovarotene. Table 10 shows the LOD & LOQ values for Palovarotene.

Robustness

The technique's robustness was evaluated by altering the organic phase and flow rate at the specified wavelength. The values acquired were presented and found to be satisfactory, indicating that the procedure remains robust. The results are presented in Table 12 & 13.

Applicability of developed method

99.79 % was the calculated mean % assay of Palovarotene respectively which was satisfactory and in good agreement with the % label claim for Palovarotene.

DISCUSSION

A QbD approach, with its emphasis on risk assessment and management, may result in the establishment of a more robust or rugged system. Critical quality attributes (CQAs) and the analytical target profile were evaluated by closely examining the detailed process for analytical QbD-based optimization parameters. The present research sought to establish an accurate, simple, precise, robust, and appropriate methodology of QbD principles for the establishment of an RP-HPLC technique for estimating Palovarotene that is more robust and performs better. Three essential elements of the RP-HPLC approach buffer pH, flow rate and ratio of MP were used in the Box-Behnken design factor screening investigations. The BBD was applied to 3 independent and dependent variables at 3 levels using the Stat Ease trial version 12.0. With water Platisil C18-EP (4.6 x 250 mm, 5µm) column and comprising of a mobile phase KH_2PO_4 (pH 3.5): ACN (50:50 ml) v/v, a 1.0 ml/min flow rate and UV range at 261 nm, the best chromatographic separation was accomplished. The interrelationships between MP, pH, and flow rate at 3 distinct levels are described by the Box–Behnken experimental design. RSM plots and statistical data were used to evaluate the retention duration and theoretical plate responses. Each independent variables data design, which included ANOVA, perturbation and contour plots, and 3D model graphs, was reviewed. In this section, we examined how each element affected the response outcome. Using the ICH Q2 R1 criteria, the established RP-HPLC technology was linear, precise, accurate, robust. The lack of any desirable peak and no change in the drug's retention time confirmed the usefulness of the established approach for quantifying Palovarotene in marketed capsules, suggesting that the method's high specification and selectivity were reached.[18-19] The current RP-HPLC method for palovarotene was in compliance with the suggested ICH recommendations. [20-22] The technique can also be utilized for quality control and laboratories evaluation aimed at the assessment of Palovarotene in the drug material and capsule formulation.

CONCLUSION

The present research aims to use analytical QbD principles to establish and optimize the RP-HPLC approach for estimating the capsule formulation of palovarotene. To achieve high resilience and improve method performance, the QbD concept has been widely used in the assessment of analytical parameters. Palovarotene has been estimated in API and marketed capsules using a precise, quick, accurate, and affordable analytical approach based on the QbD methodology.

DOE is used to construct a complete statistical analysis and design spaces for the new approach, and the method is demonstrated to be robust for a larger range of situations. The technique can also be utilized for quality control and laboratories evaluation aimed at the assessment of Palovarotene in the drug material and formulation. It can be used efficiently for research analysis in organizations, industrial QC & QA departments, and accredited analytical laboratories.

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Conflict of interest

The authors do not have any conflict of interest.

Data Availability Statement

This statement does not apply to this article.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials**.**

Author Contributions

Chandramouli Chinthaginjala: Conceptualization, Methodology; Chandrasekar Raju: Writing – Original Draft; Aruna Kumari DR: Data Collection, Analysis; Chandrasekar Raju: Writing – Review & Editing; Sivagami B: Visualization, Supervision; Meena Dravidamani**:** Project Administration; Kumanan Raghunathan: Funding Acquisition; Sivagami B: Resources, Supervision.

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