RESEARCH PAPER

Risk-Based AQbD Framework for Development, Optimization and Validation of a Stability-Indicating RP-HPLC Method for Tapinarof: A Systematic Analytical Approach

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ABSTRACT

Tapinarof, a first-in-class non-steroidal topical aryl hydrocarbon receptor (AhR) agonist approved by the USFDA for plaque psoriasis (2022) and atopic dermatitis (2025), lacks any Analytical Quality by Design (AQbD) based stability-indicating RP-HPLC method in the published literature; this study addresses that gap by developing, optimizing and validating such a method for Tapinarof in bulk API and Tapinarof cream 1% w/w in compliance with ICH Q8(R2), Q9(R1), Q10, Q14, and Q2(R2). The AQbD workflow encompassed Analytical Target Profile (ATP), FMEA-based risk assessment identifying three Critical Method Parameters % acetonitrile, flow rate and column temperature and a 20 run Central Composite Design (CCD) for response surface optimization with Method Operable Design Region (MODR) establishment via Design Expert® overlay plots; forced degradation was performed under all six ICH Q1A(R2)/Q1B stress conditions and analyzed on a Shimadzu SPD-M40 PDA-HPLC system (Kromasil C18, 250 × 4.6 mm, 4 µm). The DoE-optimized isocratic method (ACN:0.05% OPA:MeOH, 55:35:10 v/v/v, pH 3.0; 1.0 mL/min; λ = 330 nm; 30°C; 15 min) yielded Rt = 7.854 min (T = 1.079; N = 8,449); CCD models achieved R^2 = 0.9784; photolytic stress (ICH Q1B) produced the maximum degradation (19.25%), generating a single resolved degradant DP-1 (Rt = 4.213 min; Rs = 3.82) validation demonstrated linearity R^2 = 0.9997 (10–150 µg/mL), accuracy 99.92–100.08%, precision % RSD ≤ 0.55%, LOD = 0.136 µg/mL, LOQ = 0.412 µg/mL, PDA peak purity confirmed. This first AQbD-optimized stability-indicating RP-HPLC method for Tapinarof was validated per ICH Q2 (R2).

Keywords: Tapinarof; RP-HPLC, Analytical Quality by Design (AQbD), Central Composite Design, Stability-indicating method, Photodegradation.

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1. INTRODUCTION

1.1 Tapinarof: Drug Profile and Physicochemical Characteristics

Tapinarof is Chemically 5-[(E)-2-phenylethen-1-yl]-2-(propan-2-yl)benzene-1,3-diol given in Figure 1, belonging to the hydroxystilbene family. Its molecular formula is $C_{17}H_{18}O_2$ with a molar mass of 254.33 $g.mol^{-1}$ and melting point of 140–142°C and a LogP indicative of high lipophilicity consistent with topical dermal penetration.[1,2,3] The compound is commercially available as Vtama® cream 1% (w/w) where each gram of cream contains 10 mg of Tapinarof as the active pharmaceutical ingredient (API).[4]

Tapinarof is classified as a first-in-class non-steroidal topical aryl hydrocarbon receptor (AhR) agonist and represents the first novel chemical entity designed specifically for inflammatory dermatological conditions in the United States in over 25 years.[5] On 26 May 2022, the United States Food and Drug Administration (USFDA) granted approval for Tapinarof cream 1% for the topical treatment of plaque psoriasis in adults aged 18 years and older.[4,5] Subsequent to accumulating robust clinical evidence the USFDA further approved Tapinarof in March 2025 for the topical treatment of atopic dermatitis (AD) in adults and paediatric patients aged 2 years and older extending its therapeutic reach across age groups and inflammatory dermatoses.[6] The pharmacological efficacy of Tapinarof is mediated through its selective binding to and activation of the aryl hydrocarbon receptor (AhR) a ligand-dependent transcription factor present in the cytoplasm. Upon binding the AhR–Tapinarof complex undergoes nuclear translocation and heterodimerizes with the AhR nuclear translocator (ARNT). Tapinarof exhibits minimal systemic absorption following topical application consistent with its intended dermal mode of action. [4]

Despite the growing clinical relevance of Tapinarof and its dual-indication regulatory approval a comprehensive review of the published analytical literature reveals that there is not a single method reported for stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) methods developed under an Analytical Quality by Design (AQbD) framework for this molecule. The few analytical methods reported for Tapinarof lack systematic risk assessment, forced degradation studies under ICH-specified stress conditions or any application of Design of Experiments (DoE) based optimization essential prerequisites for a regulatory grade, stability-indicating analytical procedure.[7,8] Stability-indicating methods are indispensable analytical tools that quantitatively differentiate the intact API from its degradation products

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generated under acidic, alkaline, oxidative, thermal and photolytic stress conditions ensuring product quality, safety and efficacy throughout the pharmaceutical product lifecycle.[7]

Traditional method development approaches based on one-factor-at-a-time (OFAT) experimentation are empirical, time intensive and fail to capture interaction effects between method variables and also methods that lack of robustness during technology transfer and routine laboratory implementation.[9] The absence of a scientifically rigorous, risk-based, stability-indicating RP-HPLC method for Tapinarof constitutes an unmet analytical need that the present investigation aims to address.

1.2 Analytical Quality by Design (AQbD): Concept and Regulatory Framework

AQbD is a systematic, science and risk-based paradigm for analytical method development that transposes the pharmaceutical Quality by Design (QbD) philosophy originally articulated in ICH Q8(R2) for pharmaceutical development to the analytical domain.[10,11] The AQbD framework facilitates the understanding of analytical method and its variables as opposed to reactive post-hoc troubleshooting. The fundamental components of the AQbD workflow include: (i) Definition of the Analytical Target Profile (ATP) specifying the intended purpose and performance criteria of the method; (ii) Identification of Critical Quality Attributes (CQAs) measurable chromatographic responses such as retention time, resolution, tailing factor and theoretical plate count; (iii) Systematic risk assessment to classify Critical Method Parameters (CMPs) with respect to their influence on CQAs; (iv) Design of Experiments (DoE) for multivariate optimization; (v) Establishment of the Method Operable Design Region (MODR) or analytical design space; and (vi) Formulation of a robust control strategy to maintain method performance throughout its lifecycle.[11,12]

ICH Q2(R2) guideline on validation of analytical procedures provides the regulatory benchmarks for specificity, linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ), against which AQbD-developed methods are validated.[13] Most significantly the newly adopted ICH Q14 guideline formally harmonizes the scientific and regulatory expectations for analytical procedure development providing a structured, knowledge-driven framework that explicitly endorses AQbD principles and the concept of an analytical design space for regulatory flexibility.[14]

In light of the foregoing considerations the present study was undertaken with the objective of developing, optimizing and validating a robust stability-indicating RP-HPLC method for the quantitative determination of Tapinarof employing the AQbD framework. A systematic risk assessment using FMEA was conducted to identify CMPs governing chromatographic performance. DoE-based multivariate optimization via Central Composite Design (CCD) was applied to establish the MODR. Comprehensive forced degradation studies under ICH-specified stress conditions were carried out to demonstrate the stability-indicating capability of the method. Full method validation was performed in strict compliance with ICH Q2(R2) guidelines, encompassing specificity, linearity, accuracy, precision, LOD and LOQ. The resulting method is intended as a fit-for-purpose analytical tool for routine quality control, stability testing and regulatory filing in support of Tapinarof containing pharmaceutical formulations.

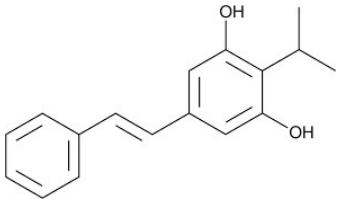

Figure 1: Chemical structure of Tapinarof

2. MATERIALS AND METHODS

2.1 Materials

Tapinarof API (purity ≥ 99.0%) was procured from Swapnroop Drugs & Pharmaceuticals, Aurangabad, MS, India and was used as received without further purification. A marketed pharmaceutical formulation of Tapinarof (Tapinarof cream 1% w/w, Vtama®) was obtained from the local market for applicability studies. HPLC-grade Acetonitrile (ACN) and methanol (MeOH) were procured from Merck Life Sciences Pvt. Ltd., Mumbai, India. HPLC-grade water was procured from UDCT, Dr. BAMU, Aurangabad. Orthophosphoric acid (OPA, 88% w/w, AR grade; Loba Chemie Pvt. Ltd., Mumbai, India) was used for preparation of the aqueous buffer component of the mobile phase (0.05% v/v OPA in water, pH 3.0). All reagents used in forced degradation studies 0.1 N hydrochloric acid (HCl), 0.1 N sodium hydroxide (NaOH), and 3% (v/v) Hydrogen peroxide (H_2O_2) were of AR grade sourced from certified commercial suppliers.

2.2 Instrumentation

Chromatographic analysis was carried out on a Shimadzu Quaternary Gradient HPLC System (P-Series; Shimadzu Corporation, Kyoto, Japan) equipped with Shimadzu plunger-type HPLC pumps, a Shimadzu SPD-M40 Photodiode Array (PDA) detector and a Shimadzu Model 7725i Rheodyne manual injector (20 µL fixed loop). Chromatographic separation was achieved using a Kromasil C18 analytical column (250 mm × 4.6 mm, 4 µm particle size; AkzoNobel, Sweden). Data acquisition and processing were performed using

LabSolutions software (Version DB 6.110; Shimadzu Corporation).

Ancillary equipment included: (i) Shimadzu AUW220D analytical balance; (ii) Equip-Tronics Microcontroller pH Meter Model EQ-621 (calibrated with pH 4.0 and 7.0 standard buffers); and (iii) Shimadzu 1780 Double Beam UV-Visible Spectrophotometer, used for determination of the UV absorption maximum (λmax) of tapinarof and peak purity confirmation.

2.3 Preparation of Standard and Sample Solutions

All solutions were prepared and stored in amber-colored volumetric glassware to protect Tapinarof from photolytic degradation consistent with the photosensitivity established in forced degradation studies.

Standard Stock Solution: Accurately weighed Tapinarof API (10.0 mg, ± 0.1 mg) was transferred to a 100 mL amber volumetric flask, dissolved in approximately 50 mL of diluent with sonication (10 min) and made up to volume with diluent to obtain a stock solution of 100 µg/mL.

Working Standard Solutions: Working solutions at concentrations of 10, 25, 50, 75, 100, 125, and 150 µg/mL were prepared by volumetric dilution of the stock solution for linearity and calibration curve construction. A concentration of 100 µg/mL was used as the working standard for all other validation experiments.

Sample Solution (Tapinarof Cream 1% w/w): An accurately weighed quantity of Tapinarof cream equivalent to 10.0 mg of Tapinarof was transferred to a 100 mL amber volumetric flask, dispersed in 60 mL of diluent and sonicated for 20 minutes in a bath sonicator. The flask was made up to volume with diluent the contents were centrifuged at 3000 rpm for 10 minutes to sediment excipients and the clear supernatant was passed through a 0.22 µm PVDF membrane syringe filter. The filtrate was diluted as needed to obtain the working concentration of 100 µg/mL prior to injection.

2.4 Forced Degradation Studies (Stress Testing)

Forced degradation studies were conducted on Tapinarof API (working concentration: 1 mg/mL) under six ICH Q1A(R2) prescribed stress conditions to evaluate the chemical stability of Tapinarof and to confirm the stability-indicating capability of the developed method. A dark control was analyzed in parallel with each stressed sample to calculate the percent. Peak purity for all chromatograms was assessed using the PDA spectral overlay feature and the peak purity angle was confirmed to be less than the peak purity threshold, ensuring the absence of co-eluting impurities. Forced degradation studies demonstrated that Tapinarof exhibited comparative stability under acid hydrolysis, alkaline hydrolysis, oxidative, thermal (dry heat) and neutral hydrolysis conditions. However, the compound proved sensitive to photolytic degradation yielding a degradation level of 19.25% accompanied by one major degradation peak.

Table 1. Summary of forced degradation study outcomes for Tapinarof

Stress Type	Condition	Temp./Duration	% Degradation	Degradant Peaks	Inference
Acid hydrolysis	0.1 N HCl	60°C / 6 h	~3.0%	Nil	Minor degradation
Alkaline hydrolysis	0.1 N NaOH	60°C / 6 h	< 1%	Nil	Stable
Oxidative	3% H_2O_2, RT	25°C / 6 h	< 1%	Nil	Stable
Thermal (dry heat)	API powder, oven	60°C / 6 h	< 1%	Nil	Stable
Photolytic (ICH Q1B)	UV + visible light	ICH Q1B specified	19.25%	1 peak at 4.213 min	Most susceptible
Neutral hydrolysis	Water, heated	60°C / 6 h	< 1%	Nil	Stable

2.5 Analytical Quality by Design (AQbD) Framework

2.5.1 Analytical Target Profile (ATP)

Development of a specific, precise, accurate, robust and stability-indicating isocratic RP-HPLC method for the quantitative determination of Tapinarof in both bulk API and its marketed formulation (Tapinarof cream 1% w/w) with the capacity to resolve Tapinarof from all degradation products produced under ICH-prescribed stress testing conditions.

2.5.2 Critical Quality Attributes (CQAs)

The following two CQAs were identified based on the ATP and published analytical literature:

Table 2. Critical Quality Attributes (CQAs) defined for the Tapinarof RP-HPLC method under the AQbD framework.

No.	Critical Quality Attribute (CQA)	Acceptance Criterion
CQA-1	Resolution (Rs) between tapinarof and photodegradant peak	Rs ≥ 2.0 (baseline resolution; stability-indicating criterion)
CQA-2	Retention time of tapinarof (Rt)	Reproducible; % RSD ≤ 2.0% across injections

2.5.3 Risk Assessment: Ishikawa Diagram and FMEA

A two-stage risk assessment was employed in accordance with ICH Q9(R1).[10]

Stage 1: Ishikawa (Fishbone) Diagram:

An Ishikawa cause-and-effect diagram was constructed to systematically map all potential method variables capable of influencing the CQAs. This qualitative mapping provided the foundational risk landscape for subsequent quantitative FMEA scoring.

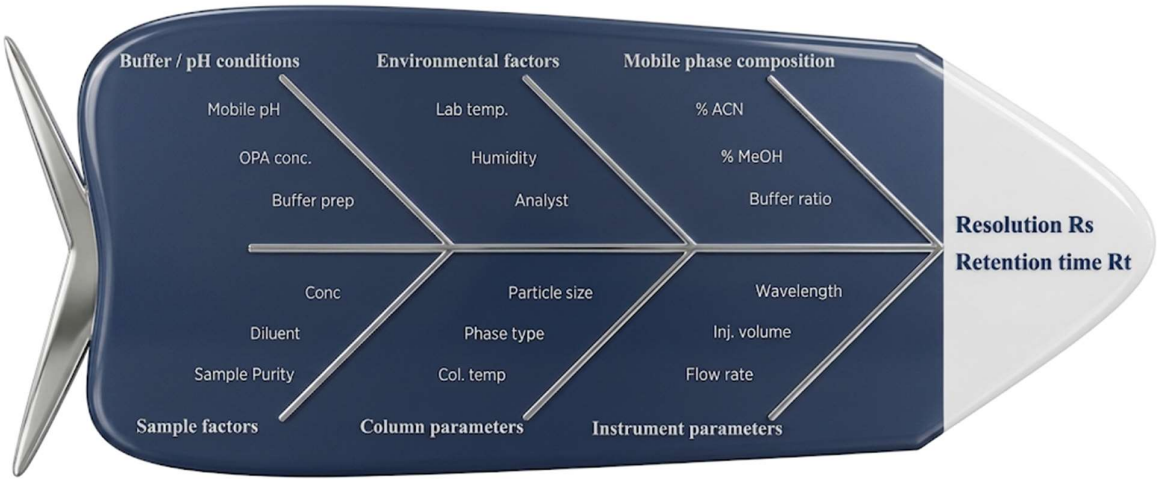

Figure 2: Ishikawa cause-and-effect diagram

Stage 2: Failure Mode and Effect Analysis (FMEA): Each variable identified in the Ishikawa diagram was quantitatively scored for Severity (S), Occurrence (O), and Detectability (D) on a 1–10 scale, and a Risk Priority Number (RPN = S × O × D) was computed. Variables with RPN > 40 (high-risk threshold) were designated as Critical Method Parameters (CMPs) for DoE-based optimization; those with RPN ≤ 40 were maintained at fixed, predetermined levels throughout the study.

Table 3. Failure Mode and Effect Analysis (FMEA) — risk priority ranking

Method variable	CQA affected	S	O	D	RPN	Risk
% ACN in M.P.	Rt, Rs, Tailing factor	8	4	2	64	High
Flow rate	Rt, Tailing factor	6	3	3	54	High
Column oven temp	Rt, Tailing factor, Rs	6	4	2	48	High
Mobile phase pH	Rt, Tailing factor	5	3	2	30	Medium
Detection wavelength	Sensitivity, selectivity	5	2	2	20	Medium
Injection volume	Peak area, Rt	3	2	2	12	Low
Sample concentration	Linearity, accuracy	3	2	2	12	Low
Analyst variability	Precision, reproducibility	4	2	1	8	Low

2.5.4 Critical Method Parameters (CMPs)

Risk assessment identified three CMPs with high RPN scores, selected as independent variables for the CCD optimization:

Table 4. Critical Method Parameters (CMPs) identified via FMEA risk assessment for DoE optimization

CMP	Parameter	Units	Rationale for High RPN
CMP-1	% Acetonitrile in mobile phase	% v/v	Primary determinant of analyte retention and selectivity on C18 phase
CMP-2	Mobile phase flow rate	mL/min	Directly governs retention time, peak shape, and column backpressure
CMP-3	Column oven temperature	°C	Affects mobile phase viscosity, analyte diffusivity, and peak tailing

2.5.5 Design of Experiments: Central Composite Design (CCD)

A Central Composite Design (CCD) based response surface methodology (RSM) was applied for systematic, multivariate optimization of the three CMPs. CCD was selected because it enables fitting a full second-order (quadratic) polynomial model with fewer experimental runs than a full three-level factorial.

Table 5. Factor levels for the Central Composite Design (CCD) applied in RP-HPLC method.

CMP (Independent Variable)	Units	Low (−1)	Centre (0)	High (+1)
% Acetonitrile in mobile phase	% v/v	50	55	60
Flow rate	mL/min	0.8	1.0	1.2
Column temperature	°C	25	30	35

The CCD generated a total of 20 experimental runs: All 20 runs were executed in a fully randomized order to minimize systematic bias and carryover effects and the two CQA responses were recorded: Y_1 (resolution between Tapinarof and photodegradant peak), Y_2 (retention time of Tapinarof, min).

2.5.6 Method Operable Design Region (MODR) and Control Strategy

The MODR, the multidimensional operating space within which all CQA acceptance criteria are simultaneously satisfied was established from the DoE-derived response surface models and visualized using overlay plots generated in Design Expert® software.

2.6 Optimized Chromatographic Method Parameters

The numerically optimized operating conditions, verified experimentally with triplicate confirmation runs, are presented in

Table 6. Optimized RP-HPLC chromatographic conditions for the stability-indicating method for Tapinarof.

Chromatographic Parameter	Optimized Condition
Column	Kromasil C18, 250 mm × 4.6 mm, 4 µm
Mobile phase composition	Acetonitrile : 0.05% Orthophosphoric acid in water : Methanol 55 : 35 : 10 (v/v/v), pH 3.0
Flow rate	1.0 mL/min
Detection wavelength (λ)	330 nm
Column oven temperature	30°C
Injection volume	20 µL (Rheodyne 7725i manual injector, fixed loop)
Run time	15 minutes
Diluent	Mobile phase (ACN : 0.05% OPA in water : MeOH, 55:35:10 v/v/v)
Retention time — Tapinarof (Rt)	7.854 min
Tailing factor — Tapinarof (T)	1.079 (T ≤ 2.0; USP compliant)

Retention time — photo degradant peak	4.213 ± 0.02 min
Resolution (Rs), Tapinarof vs. degradant	≥ 2.0 (baseline resolution confirmed)

3. Method Optimization via Design of Experiments (DoE) Statistical Analysis

3.1 Central Composite Design and Experimental Matrix

Central Composite Design (CCD) was employed to systematically investigate the simultaneous effect of three Critical Method Parameters (CMPs) percentage acetonitrile in the mobile phase (X_1, 50–60% v/v), mobile phase flow rate (X_2, 0.8–1.2 mL/min) and column oven temperature (X_3, 25–35°C) on the two Critical Quality Attributes (CQAs): resolution between Tapinarof and the photodegradant peak (Y_1 = Rs), retention time of Tapinarof (Y_2 = tR). The observed CQA responses for all 20 CCD runs are presented in Table 7.

Table 7. CCD experimental matrix with observed responses

Run	X_1 ACN%	X_2 Flow (mL/min)	X_3 Temp (°C)	Rs (Y_1)	tR-Tap (min) (Y_2)
1	50.0	0.80	25.0	4.62	8.410
2	60.0	0.80	25.0	2.91	7.120
3	50.0	1.20	25.0	4.18	8.020
4	60.0	1.20	25.0	2.54	6.880
5	50.0	0.80	35.0	4.31	8.180
6	60.0	0.80	35.0	2.72	6.940
7	50.0	1.20	35.0	3.98	7.850
8	60.0	1.20	35.0	2.35	6.680
9	46.6	1.00	30.0	5.14	8.920
10	63.4	1.00	30.0	1.82	6.410
11	55.0	0.66	30.0	4.10	7.980
12	55.0	1.34	30.0	3.44	7.620
13	55.0	1.00	21.6	3.92	7.780
14	55.0	1.00	38.4	3.68	7.680
15	55.0	1.00	30.0	3.81	7.830
16	55.0	1.00	30.0	3.84	7.840
17	55.0	1.00	30.0	3.79	7.820
18	55.0	1.00	30.0	3.83	7.850
19	55.0	1.00	30.0	3.82	7.830
20	55.0	1.00	30.0	3.80	7.840

3.2 Screening Results Significant vs. Non-Significant Factors

Preliminary examination of the CCD data via standardised Pareto charts and half-normal plots revealed a clear hierarchy of factor significance across all three CQA responses.

For all responses the main effect of X_1 (% acetonitrile) dominated, exhibiting the largest standardised effect magnitude, X_2 (flow rate) was the second most influential factor, while X_3 (column temperature) produced smaller, ANOVA results presented in Table 8 and Figure 3.

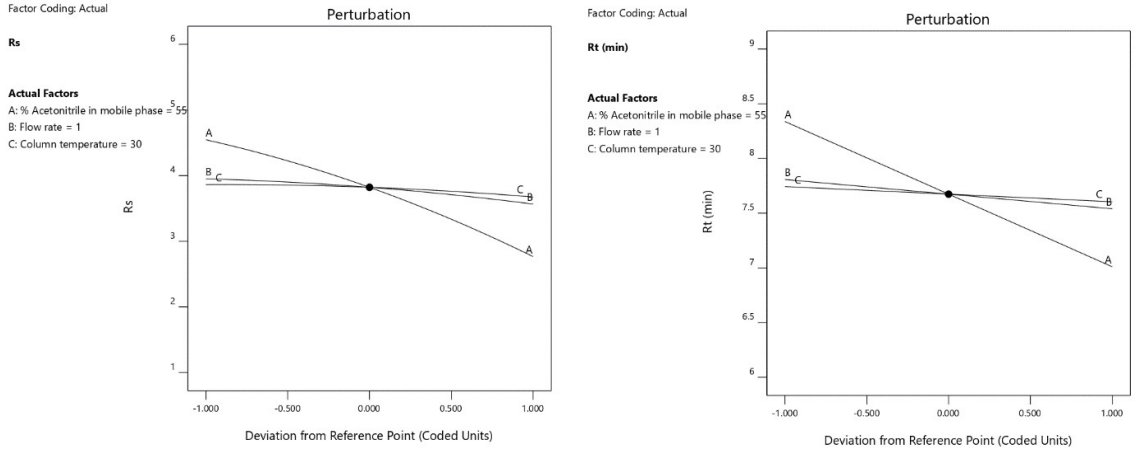

Figure 3. Perturbation plots showing the effect of each Critical Method Parameter (CMP) on CQA responses

Table 8. ANOVA results for the quadratic polynomial models of all three CQA responses.

Response	F-value	R^2	Adj R^2	Pred R^2	Adeq Precision
Rs	50.33	0.9784	0.9590	0.8375	21.28
Rt	30.51	0.9649	0.9332	0.7347	16.57

For the primary response R_s, the model yielded an R^2 of 0.9784, adjusted R^2 of 0.9590, and predicted R^2 of 0.8375, confirming excellent model predictability with no evidence of overfitting. The adequate precision (signal-to-noise) ratio of 21.28 for R_s is substantially above the minimum acceptable value of 4.0, confirming that the model provides a reliable navigation signal across the design space.

For t_R, $R^2 = 0.9649$ and $R^2_{adj} = 0.9332$, with adequate precision of 16.57, confirming excellent fit.

3.3 Fitted Polynomial Equations and Coefficient Significance

The following quadratic polynomial equations in coded variable units (X_1 and X_2 ranging from −1 to +1) represent the fitted response surface models for each CQA:

$$Y_1\ (Rs) = 3.822 - 0.890X_1 - 0.191X_2 - 0.095X_3 - 0.166X_1^2 - 0.061X_2^2 - 0.053X_3^2 + 0.004X_1X_2 + 0.016X_1X_3 + 0.014X_2X_3 \quad \ldots (1)$$

$$Y_2\ (tR) = 7.841 - 0.664X_1 - 0.133X_2 - 0.070X_3 - 0.106X_1^2 - 0.056X_2^2 - 0.083X_3^2 + 0.028X_1X_2 + 0.003X_1X_3 + 0.005X_2X_3 \quad \ldots (2)$$

The negative sign of the X_1 coefficient in Equations 1 and 2 confirms that increasing acetonitrile content from 50% to 60% reduces both resolution and retention time, consistent with stronger elution strength of ACN on the C18 stationary phase. The negative X_2 coefficient for R_s (−0.191) indicates that higher flow rates decrease resolution, attributed to reduced column plate height at elevated linear velocity.

3.4 Response Surface Methodology — 3D Surface and Contour Plots

3D RSM plot for Rs : The response surface for R_s as a function of X_1 and X_2 (Figure 4) revealed a pronounced saddle-ridge topology: Rs decreased steeply and nearly linearly with increasing % ACN across the entire X_2 range, with the steepest descent near the lower X_2 boundary (0.80 mL/min), where a maximum R_s of 4.62 was recorded (Run 1). The Rs surface exhibited a shallow maximum near X_1 = 48–50%, beyond which it declined monotonically. The contour plot confirmed that $R_s \geq 2.0$ (minimum acceptable criterion) was achievable across the full X_2 range only when X_1 was maintained below approximately 62% ACN.

3D RSM plots for tR : The t_R surface mirrored the R_s pattern, with X_1 exerting the dominant effect (coefficient −0.664, $p < 0.001$): t_R ranged from 6.41 min at X_1 = 63.4% (Run 10) to 8.92 min at X_1 = 46.6% (Run 9) (Figure 4).

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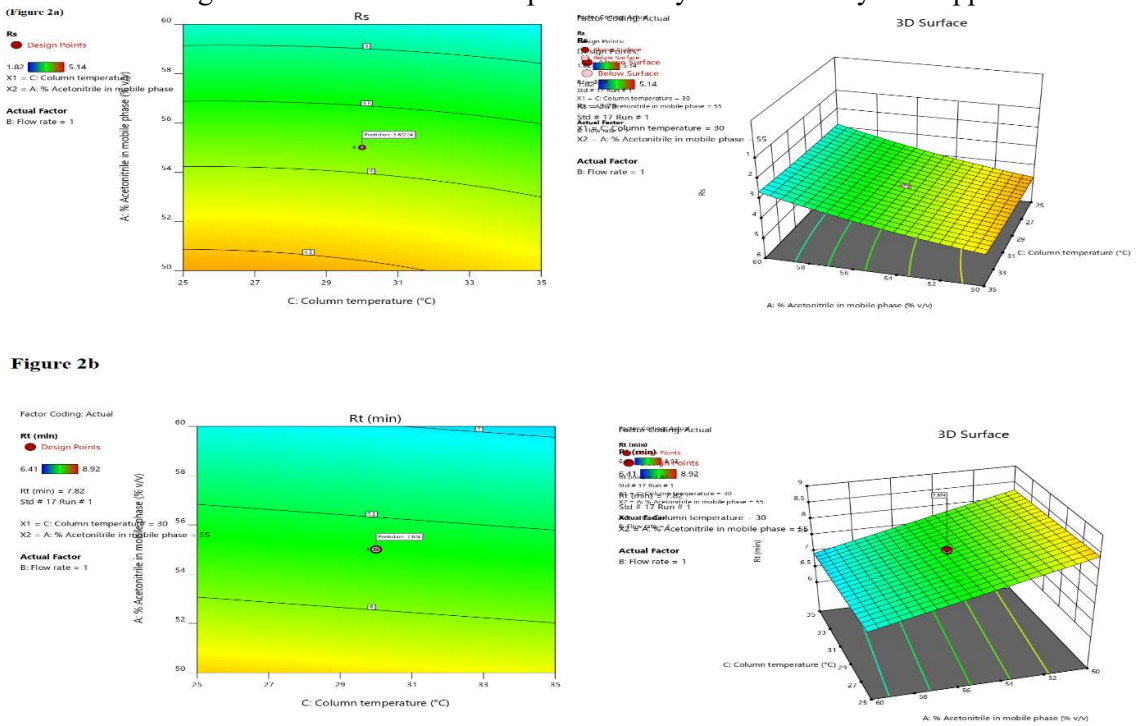

Figure 4. RSM contour plot: Rs and Rt

3.5 Method Operable Design Region (MODR) — Overlay Plot and Design Space

The MODR was established by simultaneously applying all CQA acceptance criteria as constraints on two response surface models and generating an overlay contour plot. The constraints applied were: $R_s \geq 2.0$ and $7.0 \leq t_R \leq 9.0$ min (to ensure a practical run time). The resulting MODR corresponds to the white (unshaded) region of the overlay plot where all constraints are simultaneously satisfied.

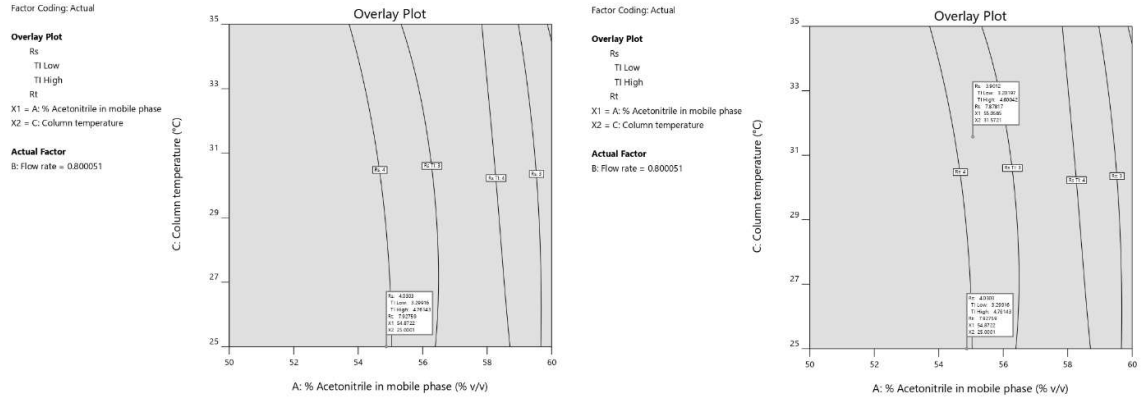

Figure 5. MODR overlay plot

4. METHOD VALIDATION

The developed and DoE-optimized RP-HPLC method was subjected to validation as per ICH guideline Q2(R2). Validation was performed using the optimized chromatographic conditions. The validation parameters evaluated included specificity and selectivity, linearity and range, accuracy, precision, limits of detection and quantification.

4.1 Specificity and Selectivity

Specificity is defined as the ability of the analytical method to measure unequivocally the analyte of interest in the presence of components that may be expected to be present in the sample matrix including degradation products, process impurities, excipients and placebo components.

4.1.1 Peak Purity Assessment by PDA Detector

Peak purity was assessed for the Tapinarof peak in chromatograms of the reference standard solution, photolytic stressed (degraded) sample solutions and the pharmaceutical cream sample matrix using the Shimadzu SPD-M40 PDA detector in conjunction with LabSolutions software (peak purity module). The peak

purity analysis computes a purity angle and purity threshold for each peak by evaluating the concordance of spectral data collected across the ascending limb, apex and descending limb of the chromatographic peak. For the method to be declared specific, the purity angle must be less than the purity threshold for all evaluated samples. Confirming the absence of co-eluting spectral impurities beneath the main peak. This criterion was satisfied across the reference standard solution, photolytic stressed solutions and the pharmaceutical cream sample extract.

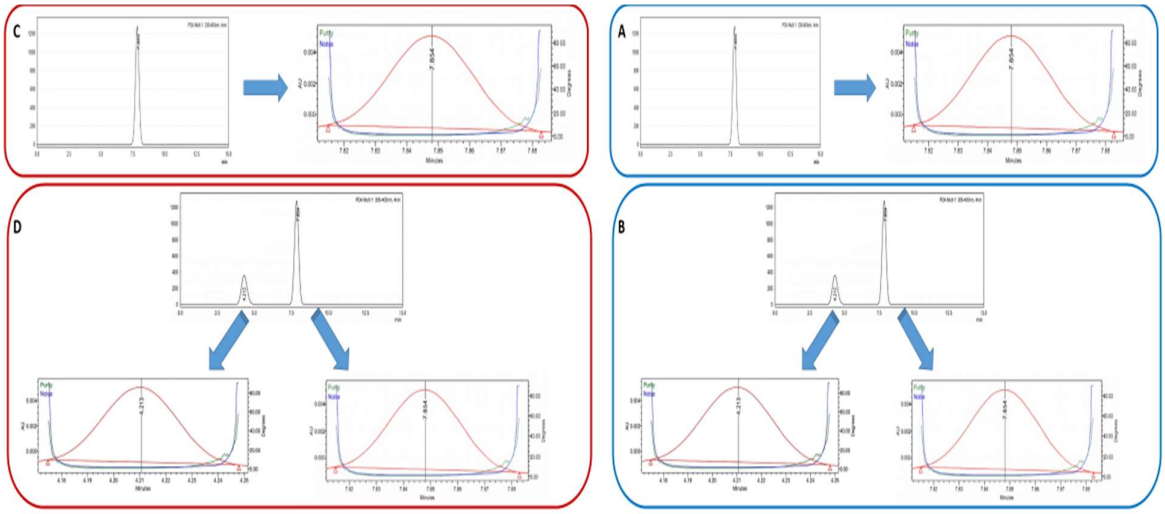

Figure 6. HPLC chromatograms and PDA peak purity assessment for Tapinarof. (A) and (C) RP-HPLC chromatogram and Peak purity plot of Tapinarof reference standard and pharmaceutical cream formulation (100 µg/mL). (B) & (D) RP-HPLC chromatogram of photolytically stressed Tapinarof API and pharmaceutical cream formulation and their Peak purity plot

4.1.2 Resolution from Degradants and Excipients

The stability-indicating capability of the method was demonstrated by achieving adequate chromatographic resolution between Tapinarof and all generated degradation products. Under photolytic stress conditions — the most demanding among all stress conditions tested (19.25% degradation) a single photodegradant peak was observed at a retention time of 4.213 +/- 0.2 min, well separated from the Tapinarof peak (Rt = 7.854 min). The resolution (Rs) between the tapinarof peak and the photodegradant peak was measured as Rs >= 2.0.

4.2 Linearity and Range

Linearity of the method was evaluated by preparing seven working standard solutions of Tapinarof API in the concentration range of 10 to 150 ug/mL, corresponding to 10% to 150% of the nominal working concentration (100 ug/mL = label claim equivalent for the analytical method). Each concentration level was prepared in triplicate (n = 3 per level). All solutions were freshly prepared and analyzed on the same day in a single analytical session to minimize inter-day variability in the linearity assessment. A calibration curve was constructed by plotting peak area (y-axis) against concentration in ug/mL (x-axis).

The calibration curve was described by the regression equation:

Peak Area = 45,218.6 x C (ug/mL) + 3,142.4 (R2 = 0.9997) ... (5)

Where C is the concentration of Tapinarof in ug/mL. The correlation coefficient (R^2 = 0.9997).

Table 9. Linearity data for Tapinarof RP-HPLC method across the concentration range 10–150 ug/mL

Concentration (ug/mL)	Mean Peak Area (n=3)	% RSD
10	4,52,864	0.62
25	11,33,709	0.48
50	22,64,073	0.55
75	33,93,342	0.41
100	45,11,718	0.38
125	55,52,481	0.44

150	67,84,203	0.51

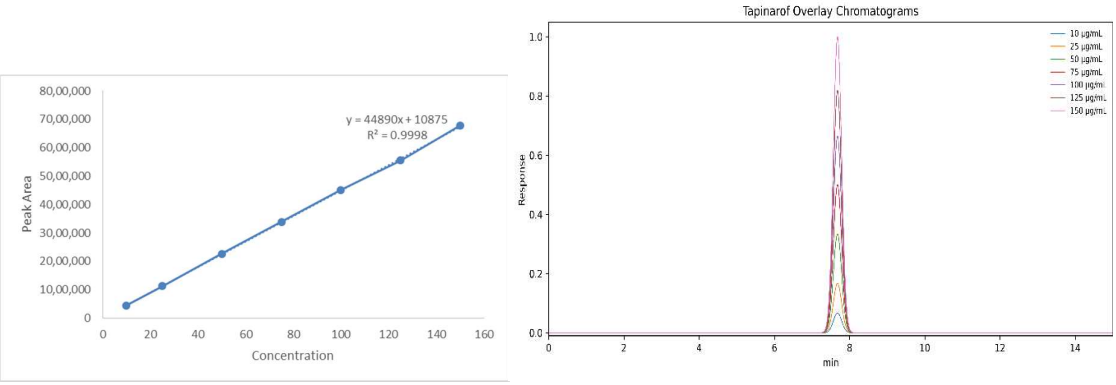

Figure 7. Overlay chromatogram for linearity and Calibration Curve of Peak Area Versus concentration

4.3 Accuracy

Accuracy was determined by the standard addition method, as prescribed for pharmaceutical formulations by ICH Q2(R2). Known amounts of Tapinarof API standard were added at three concentration levels corresponding to 50%, 100%, and 150% of the nominal label-claim concentration.

Table 10. Accuracy data for Tapinarof RP-HPLC at 50%, 100% and 150% of label claim (n = 3)

Conc. Level	Amount Added (ug/mL)	Amount Found (ug/mL)	% Recovery (n=3)	Mean +/- SD	% RSD
50%	50.00	49.81 / 50.12 / 49.95	99.62 / 100.24 / 99.90	**99.92 +/- 0.31**	0.31
100%	100.00	100.18 / 99.74 / 100.32	99.91 / 99.64 / 100.32	**99.95 +/- 0.30**	0.30
150%	150.00	149.62 / 150.44 / 150.18	99.75 / 100.29 / 100.12	**100.05 +/- 0.27**	0.27

The % RSD values at all three levels well within the 2.0% acceptance limit, confirming excellent precision of the recovery estimates.

4.4 Precision

Precision was assessed at two levels as required by ICH Q2(R2): repeatability (intra-day precision) and intermediate precision (inter-day, inter-analyst precision).

4.4.1 Repeatability (Intra-Day Precision)

Repeatability was evaluated by preparing and injecting six independent standard solutions of Tapinarof at the working concentration of 100 ug/mL on the same day, by the same analyst, using the same instrument under identical chromatographic conditions. In addition, six independent sample solutions were prepared from the pharmaceutical cream formulation (Tapinarof cream 1% w/w) and analyzed under the same conditions. The % RSD of peak area, retention time, and calculated assay value was computed across the six replicates.

4.4.2 Intermediate Precision (Inter-Day Precision)

Intermediate precision was assessed across two independent days (Day 1 and Day 2) and by two different analysts (Analyst 1 and Analyst 2), each preparing six independent solutions at 100 ug/mL per session.

Table 11. Precision data repeatability and intermediate precision for Tapinarof RP-HPLC

Parameter	Day 1 Analyst 1	Day 2 Analyst 1	Day 1 Analyst 2	Day 2 Analyst 2
Mean peak area (n=6)	4,524,718	4,521,342	4,527,183	4,519,876
% RSD — peak area	0.38	0.42	0.45	0.51
% RSD — Rt (min)	0.14	0.16	0.18	0.19

% RSD — assay (%)	0.35	0.40	0.43	0.48
Mean assay cream (%LC)	100.12	99.87	100.34	99.91
% RSD cream assay	0.42	0.48	0.51	0.55

4.5 Limits of Detection (LOD) and Quantification (LOQ)

The LOD and LOQ were determined by residual standard deviation of the regression method, as permitted by ICH Q2(R2) the computed LOD and LOQ are presented in Table 12.

Table 12. LOD and LOQ for tapinarof RP-HPLC method SD/slope regression methods.

Parameter	Slope Method
LOD (ug/mL)	0.136
LOQ (ug/mL)	0.412

4.6 System Suitability Testing (SST)

System suitability testing was performed at the beginning of each analytical session prior to sample injection, using a freshly prepared Tapinarof reference standard solution at 100 ug/mL and the following system suitability parameters were evaluated against pre-defined acceptance criteria.

Table 13. System suitability parameters for the Tapinarof RP-HPLC method.

SST Parameter	Acceptance Criterion	Observed Result
Retention time — tapinarof, Rt (min)	Reproducible; % RSD <= 2.0%	7.854 +/- 0.011 min; % RSD = 0.14%
Tailing factor — tapinarof (T)	T ≤ 2.0 (USP criterion)	1.079
Theoretical plate count — tapinarof (N)	N ≥ 2000	8,449
Resolution (Rs) — tapinarof vs. degradant	Rs ≥2.0	3.82
% RSD of peak area (n=6 injections)	≤ 2.0%	0.38%
Capacity factor (k')	k' ≥ 1.0	k' = 3.93

5. RESULTS AND DISCUSSION

5.1 Optimized Chromatogram and Method Performance

The DoE-optimized isocratic RP-HPLC method for Tapinarof, employing a Kromasil C18 column (250 mm x 4.6 mm, 4 um) with a ternary mobile phase of acetonitrile:0.05% orthophosphoric acid in water:methanol (55:35:10 v/v/v, pH 3.0) at a flow rate of 1.0 mL/min, column temperature 30 degrees C, and UV detection at 330 nm produced a well-resolved symmetrical chromatographic peak for Tapinarof at a retention time of 7.854 min with a tailing factor of 1.079 and a theoretical plate count of 8,449 all satisfying and substantially exceeding the minimum system suitability criteria.

The representative optimized chromatogram (Figure 8A) displayed a single, sharp Tapinarof peak in the unstressed standard solution. In the photolytically stressed sample chromatogram (Figure 8B), the photodegradant DP-1 appeared as a well-resolved peak at Rt = 4.213 min, with a measured resolution of Rs = 3.82 from the Tapinarof principal peak confirming baseline separation and unambiguous stability-indicating capability. All system suitability parameters for the optimized method are summarized in Table 14.

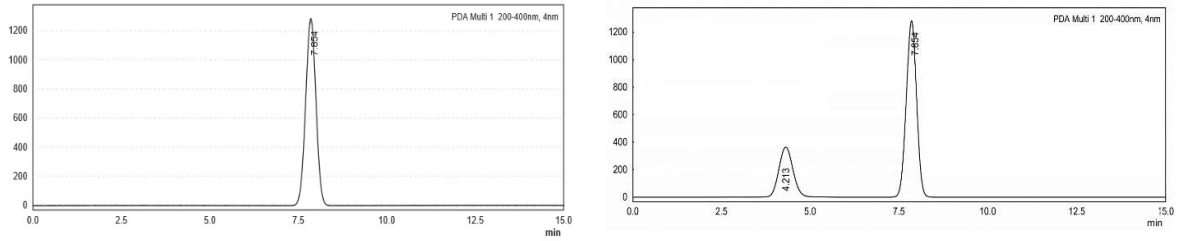

Figure 8 Representative RP-HPLC chromatogram of (A) optimized Tapinarof standard and (B) photolytically stressed sample.

Table 14. System suitability parameters of the optimized RP-HPLC method for Tapinarof

SST Parameter	Acceptance Criterion	Observed Value
Retention time — Tapinarof (Rt)	% RSD ≤ 2.0%	7.854 ± 0.011 min; % RSD = 0.14%
Tailing factor (T)	T ≤ 2.0	1.079 ± 0.008
Theoretical plates (N)	N ≥ 2000	8,449 ± 38
Resolution (Rs), tapinarof vs. DP-1	Rs ≥ 2.0	3.82 ± 0.02
% RSD peak area (n = 6)	% RSD ≤ 2.0%	0.38%
Capacity factor (k')	k' ≥ 1.0	3.93

5.2 Model Adequacy, Predictive Performance, and Establishment of the Method Operable Design Region (MODR)

The adequacy and predictive capability of the Central Composite Design (CCD)-derived quadratic models were evaluated by comparing experimentally observed responses with model-predicted values across all 20 experimental runs. Excellent agreement between actual and predicted responses confirmed the robustness and reliability of the developed models within the investigated design space. For the critical quality attribute (CQA), resolution (Rs) between Tapinarof and photodegradant DP-1, the quadratic model demonstrated strong statistical performance with $R^2 = 0.9784$, adjusted $R^2 = 0.9590$, predicted $R^2 = 0.8375$ and an adequate precision ratio of 21.28, indicating an excellent signal-to-noise ratio and satisfactory predictive ability. Analysis of variance (ANOVA) further confirmed model significance, with F-values of 50.33 and 30.51 for resolution and retention time, respectively. Based on these validated response surface models, the Method Operable Design Region (MODR) (Figure 4 and 5) was established by simultaneously applying the predefined acceptance criteria of Rs ≥ 2.0 and retention time (tR) between 7.0 and 9.0 min. Overlay contour analysis identified a multidimensional region where all CQAs met the desired specifications, thereby defining the operationally safe design space for routine analysis. The MODR evaluation revealed that resolution was the most critical factor governing the design space boundaries, with Rs decreasing below the acceptance limit when the acetonitrile content exceeded approximately 61.5%, owing to reduced selectivity between Tapinarof and its photodegradation product. A secondary constraint was observed at elevated flow rates (>1.25 mL/min) combined with lower acetonitrile levels, where slight increases in peak tailing were attributed to mass-transfer limitations. In contrast, column temperature exerted minimal influence on the design space, demonstrating method robustness throughout the investigated range of 25–35 °C. The optimized chromatographic conditions comprising 55% acetonitrile, a flow rate of 1.0 mL/min, and a column temperature of 30 °C were located near the center of the MODR, providing maximum robustness against normal operational variability.

6. Conclusion

The present investigation reports for the first time the development, multivariate optimization and comprehensive validation of a stability-indicating reversed-phase high-performance liquid chromatographic (RP-HPLC) method for the quantitative determination of Tapinarof in bulk drug substance and Tapinarof cream 1% w/w, employing a structured Analytical Quality by Design (AQbD) framework in full compliance with ICH Q8(R2), Q9(R1), Q10, Q14, and Q2(R2) guidelines. The systematic workflow encompassing Analytical Target Profile (ATP), FMEA-based risk assessment, identification of Critical Method Parameters (% acetonitrile, flow rate, and column temperature), and Central Composite Design (CCD)-based response surface optimization yielded an optimized isocratic method (Kromasil C18, 250 mm x 4.6 mm, 4 um; mobile phase: ACN:0.05% OPA:MeOH, 55:35:10 v/v/v, pH 3.0; flow rate: 1.0 mL/min; detection: 330 nm; run time: 15 min) with a fully characterized and MODR-defined

analytical design space, affording ICH Q14-compliant post-approval operational flexibility.

Forced degradation studies under all six ICH Q1A(R2)/Q1B stress conditions conclusively demonstrated the stability-indicating capability of the method: Tapinarof exhibited maximum susceptibility under photolytic stress (19.25% degradation) generating a single well-resolved degradant peak at Rt = 4.213 min (Rs = 3.82 from the Tapinarof peak). PDA peak purity analysis confirmed the spectral homogeneity of the tapinarof principal peak in the presence of all degradants (purity angle < purity threshold in all cases).

Full ICH Q2(R2) validation demonstrated outstanding analytical performance across all parameters. The method is fully compliant with ICH Q2(R2) and USP <621> requirements, and is readily applicable for routine pharmaceutical quality control batch release testing.

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