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# Quality by Design-Assisted RP-HPLC Method Development and Validation for Quantitative Estimation of Baloxavir Marboxil in Bulk and Pharmaceutical Dosage Forms

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**ABSTRACT**

**Background:** Baloxavir marboxil is a novel cap-dependent endonuclease inhibitor indicated for the treatment of acute uncomplicated influenza in adults and pediatric patients. Accurate and reliable analytical methods are essential for quality control of this antiviral agent in pharmaceutical dosage forms. **Objective:** To develop and validate a simple, precise, accurate, and robust reversed-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative estimation of baloxavir marboxil in bulk drug and pharmaceutical dosage forms using a Quality by Design (QbD) approach. **Methods:** Chromatographic optimization was performed using a Box–Behnken design (BBD), evaluating the effect of buffer pH (2.0–3.0), organic phase ratio (50–60% v/v), and flow rate (0.8–1.0 mL/min) on retention time and tailing factor. Optimized conditions employed a PLATISIL C18 column (4.6 × 250 mm, 5 µm) with a mobile phase of methanol and $KH_2PO_4$ buffer (pH 4.5) in a ratio of 60:40 v/v, at a flow rate of 1 mL/min, with UV detection at 247 nm. Method validation was conducted as per ICH Q2(R1) guidelines. **Results:** The method demonstrated excellent linearity over the concentration range of 10–50 µg/mL ($R^2 = 0.9998$). Precision studies yielded %RSD values below 1%, and accuracy was confirmed with recovery values in the range of 98–102%. The limit of detection (LOD) and limit of quantitation (LOQ) were 0.37 ppm and 1.25 ppm, respectively. System suitability parameters, including tailing factor (<2.0) and theoretical plates (>2000), complied with acceptance criteria. **Conclusion:** The QbD-assisted RP-HPLC method is specific, precise, accurate, reproducible, and robust, rendering it suitable for routine quality control, stability assessment, and regulatory analysis of baloxavir marboxil in pharmaceutical preparations.

**Keywords:** Baloxavir marboxil, RP-HPLC, Method validation, ICH Q2(R1), Quality by Design, Box–Behnken design

## 1. INTRODUCTION

Influenza virus infection continues to represent a significant global health burden, causing substantial morbidity, mortality, and socioeconomic loss, particularly among high-risk populations including the elderly, immunocompromised individuals, and young children [1]. The development of efficacious antiviral agents with novel mechanisms of action is therefore a public health priority. Baloxavir marboxil (BXM), marketed under the trade name Xofluza®, is a first-in-class antiviral prodrug that is metabolized in vivo to its active form, baloxavir acid (BXA), which selectively inhibits the cap-dependent endonuclease (CEN) activity of the influenza polymerase acidic (PA) subunit [2, 3].

Unlike neuraminidase inhibitors such as oseltamivir, BXM acts at an earlier stage of the viral replication cycle, providing a distinct mechanism that circumvents pre-existing neuraminidase resistance and reduces viral shedding more rapidly [4]. Approved by the U.S. Food and Drug Administration (FDA) in 2018, BXM is indicated for the treatment of acute uncomplicated influenza in patients aged twelve years and older and for post-exposure prophylaxis [5].

Quality by Design (QbD) is a systematic, science- and risk-based pharmaceutical development approach advocated by regulatory agencies including the FDA and the International Council for Harmonization (ICH) through guidance documents such as ICH Q8(R2), Q9, and Q10 [6]. Application of QbD to analytical method development facilitates a thorough understanding of method variables, identification of critical method attributes (CMAs), and robust optimization through design of experiments (DoE). Box–

Behnken design (BBD), a widely employed response surface methodology (RSM), allows efficient evaluation of multiple factors and their interactions with a reduced number of experimental runs [7].

A review of published literature reveals several analytical methods for the determination of BXM, including stability-indicating RP-HPLC methods [8], LC–MS/MS bioanalytical methods [9–11], and fluorometric techniques [12]. However, very few reports have integrated a QbD framework into the RP-HPLC method development for BXM in pharmaceutical dosage forms, and none have employed BBD-based optimization in conjunction with comprehensive ICH Q2(R1) validation for routine quality control purposes.

The present investigation, therefore, aimed to develop and validate a simple, sensitive, and robust RP-HPLC method for the quantitative estimation of baloxavir marboxil in bulk drug substance and tablet dosage form, employing QbD principles to systematically optimize chromatographic conditions and ensure method performance across the intended operating space.

## 2. MATERIALS AND METHODS

### *2.1. Drug and Chemicals*

Baloxavir marboxil working standard (purity ≥99.0%) was procured from MSN Laboratories Pvt. Ltd. (Hyderabad, India). Potassium dihydrogen phosphate ($KH_2PO_4$, AR grade) was obtained from FINAR Chemical Ltd. (Ahmedabad, India). HPLC-grade methanol, acetonitrile, and purified water were supplied by Standard Solutions Ltd. Hydrochloric acid (HCl), hydrogen peroxide ($H_2O_2$), and sodium hydroxide (NaOH) for forced degradation studies were purchased from Merck (Mumbai, India). All chemicals and reagents used were of analytical or HPLC grade.

### *2.2. Instruments and Equipment*

The following instruments were employed in this study:

**Table 1: Instruments Used in the Study**

| S. No. | Instrument | Make / Model |
|---|---|---|
| 1 | High-Performance Liquid Chromatograph | Waters Alliance HPLC System (2695 Separation Module, 2487 UV Detector); Empower Software |
| 2 | UV/Vis Spectrophotometer | LABINDIA UV 3000⁺ |
| 3 | pH Meter | Adwa AD 1020 |
| 4 | Analytical Balance | Afcoset ER-200A (±0.01 mg) |
| 5 | Ultrasonic Water Bath | Loba Chemie / Standard |
| 6 | Volumetric Glassware | Borosil (Class A) |

**Table 2: Chemicals and Reagents Used in the Study**

| S. No. | Chemical / Reagent | Supplier / Grade |
|---|---|---|
| 1 | Baloxavir Marboxil (Working Standard) | MSN Laboratories Pvt. Ltd., India |
| 2 | Potassium Dihydrogen Phosphate ($KH_2PO_4$) | FINAR Chemical Ltd., AR Grade |
| 3 | Methanol (HPLC grade) | Standard Solutions Ltd. |
| 4 | Water (HPLC grade) | Standard Solutions Ltd. |
| 5 | Acetonitrile (HPLC grade) | Standard Solutions Ltd. |
| 6 | HCl, $H_2O_2$, NaOH (for forced degradation) | Merck, AR Grade |

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### *2.3. Drug Profile*

Baloxavir marboxil (molecular formula: $C_{27}H_{23}FN_3O_7PS$; molecular weight: 571.52 g/mol) is a bicyclic acid prodrug that undergoes hydrolysis to form the active metabolite, baloxavir acid (BXA). BXA inhibits the cap-snatching mechanism of the influenza RNA-dependent RNA polymerase by targeting the PA subunit endonuclease domain, thereby inhibiting viral replication. The compound is practically insoluble in water and is available as 40 mg and 80 mg film-coated tablets.

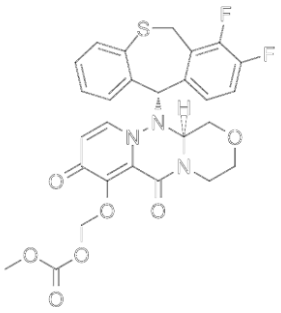


**Fig. 1: Chemical Structure of Baloxavir Marboxil**

### *2.4. Quality by Design (QbD) Approach*

A QbD framework was adopted for systematic method development. The Analytical Target Profile (ATP) was defined as a robust RP-HPLC method suitable for routine quantification of BXM with precision, accuracy, and specificity. Critical method attributes (CMAs) identified were retention time (Rt) and tailing factor (Tf). Critical method parameters (CMPs) selected for optimization using Box–Behnken Design (BBD) were: (A) buffer pH (2.0–3.0), (B) organic phase ratio (50–60% v/v), and (C) flow rate (0.8–1.0 mL/min). A total of 17 experimental runs were generated by Design-Expert® software.

**Table 3: Box–Behnken Design Matrix with Observed Responses for Baloxavir Marboxil**

| Std | Run | A: Buffer pH | B: Mobile Phase Ratio (%) | C: Flow Rate (mL/min) | Rt (min) | Tailing Factor |
|---|---|---|---|---|---|---|
| 2 | 1 | 3.00 | 50.00 | 0.90 | 3.140 | 0.860 |
| 13 | 2 | 2.50 | 55.00 | 0.90 | 3.144 | 0.849 |
| 11 | 3 | 2.50 | 50.00 | 1.00 | 3.143 | 0.849 |
| 16 | 4 | 2.50 | 55.00 | 0.90 | 3.144 | 0.847 |
| 5 | 5 | 2.00 | 55.00 | 0.80 | 3.140 | 0.848 |
| 10 | 6 | 2.50 | 60.00 | 0.80 | 3.143 | 0.849 |
| 17 | 7 | 2.50 | 55.00 | 0.90 | 3.142 | 0.849 |
| 1 | 8 | 2.00 | 50.00 | 0.90 | 3.140 | 0.848 |
| 12 | 9 | 2.50 | 60.00 | 1.00 | 3.145 | 0.850 |
| 7 | 10 | 2.00 | 55.00 | 1.00 | 3.144 | 0.849 |
| 6 | 11 | 3.00 | 55.00 | 0.80 | 3.142 | 0.848 |
| 8 | 12 | 3.00 | 55.00 | 1.00 | 3.141 | 0.860 |
| 15 | 13 | 2.50 | 55.00 | 0.90 | 3.142 | 0.849 |
| 3 | 14 | 2.00 | 60.00 | 0.90 | 3.144 | 0.850 |
| 9 | 15 | 2.50 | 50.00 | 0.80 | 3.144 | 0.849 |
| 4 | 16 | 3.00 | 60.00 | 0.90 | 3.140 | 0.848 |
| 14 | 17 | 2.50 | 55.00 | 0.90 | 3.142 | 0.847 |

### *2.5. Optimized Chromatographic Conditions*

Following statistical analysis of the BBD results, the chromatographic conditions were optimized as follows:

Column: PLATISIL C18 (4.6 × 250 mm, 5 µm)

Mobile phase: Methanol : $KH_2PO_4$ buffer (pH 4.5) = 60:40 v/v

Flow rate: 1.0 mL/min

Detection wavelength: 247 nm

Injection volume: 20 µL

Column temperature: Ambient (25 ± 2°C)

Run time: 10 min

### *2.6. Preparation of Buffer and Mobile Phase*

$KH_2PO_4$ buffer (pH 4.5): 0.1 mL of formic acid was dissolved in 1000 mL of HPLC-grade water and the pH was adjusted to 4.5 ± 0.05 using 0.1 M sodium hydroxide solution. The buffer was filtered through a 0.45 µm nylon membrane filter under vacuum and degassed in an ultrasonic water bath for 10 min.

Mobile phase: $KH_2PO_4$ buffer (pH 4.5) and HPLC-grade methanol were mixed in a ratio of 40:60 (v/v), filtered through a 0.45 µm membrane filter, and degassed for 10 min prior to use.

Diluent: Methanol : $KH_2PO_4$ buffer (pH 4.5) in a 60:40 v/v ratio was used as the diluent throughout the study.

### *2.7. Preparation of Standard and Sample Solutions*

Standard solution: Accurately weighed 25 mg of baloxavir marboxil working standard was transferred to a 25 mL volumetric flask. Approximately 15 mL of diluent was added, and the solution was sonicated for 10 min to dissolve completely. The volume was made up to the mark with diluent to obtain a stock solution of 1000 µg/mL. From this stock, 0.3 mL was transferred to a 10 mL volumetric flask and diluted to volume with diluent to yield a working standard solution of 30 µg/mL.

Sample solution: An equivalent weight of tablet powder corresponding to 25 mg of BXM was accurately weighed and transferred to a 25 mL volumetric flask. The procedure followed was identical to that of the standard solution preparation.

### *2.8. System Suitability*

System suitability was assessed by injecting the standard solution six times and evaluating theoretical plates ($N \geq 2000$) and tailing factor ($Tf \leq 2.0$) for the BXM peak, as per ICH guidelines.

### *2.9. Method Validation*

The developed RP-HPLC method was validated in accordance with ICH Q2(R1) guidelines for the following parameters:

**Linearity:** Standard solutions were prepared at six concentration levels (10, 20, 30, 40, and 50 µg/mL) and injected in triplicate. A calibration curve was plotted and regression statistics were calculated.

**Precision:** Repeatability (intra-day precision) was assessed by six replicate injections of the working standard solution at 30 µg/mL on the same day. Intermediate precision (inter-day precision) was evaluated on three consecutive days at the same concentration.

**Accuracy:** Recovery studies were conducted by spiking pre-analyzed tablet powder with known quantities of the standard drug at three concentration levels (80%, 100%, and 120% of the label claim) in triplicate.

**Specificity:** The specificity of the method was assessed by comparing chromatograms of blank (diluent), standard, and sample solutions.

**Sensitivity (LOD and LOQ):** The limit of detection (LOD) and limit of quantitation (LOQ) were determined by the signal-to-noise (S/N) ratio method, targeting S/N ratios of approximately 3:1 and 10:1, respectively.

**Robustness:** The influence of deliberate small changes in chromatographic conditions—namely flow rate (±0.1 mL/min), mobile phase ratio (±5%), and buffer pH (±0.2 units)—on system suitability parameters was evaluated.

**Ruggedness**: Method ruggedness was assessed by determining %RSD when the method was performed by two different analysts on different instruments under the same conditions.

### *2.10. Assay Calculation*

The percentage assay of baloxavir marboxil was calculated using the following formula:

$$\%\,Assay = \frac{AT}{AS} * \frac{WS}{DS} * \frac{DT}{WT} * \frac{Average\ weight}{Label\ Claim} * \frac{P}{100} * 100$$

*Where: AT = Mean peak area from sample preparation; AS = Mean peak area from standard preparation; WS = Weight of working standard (mg); P = Percentage purity of working standard; LC = Label claim (mg/tablet).*

## 3. RESULTS

### *3.1. QbD Optimization – Statistical Analysis*

The Box–Behnken Design generated 17 experimental runs. The chromatographic responses—retention time (Rt) and tailing factor (Tf)—were subjected to ANOVA using Design-Expert® software. The analysis of variance tables for Rt and Tf are presented in Tables 4 and 5, respectively.

**Table 4: ANOVA for Response 1 – Retention Time (Rt)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | Remarks |
|---|---|---|---|---|---|---|
| Mean vs Total | 12.28 | 1 | 12.28 | – | – | – |
| Linear vs Mean | 8.975E-005 | 3 | 2.992E-005 | 2.57 | 0.0990 | – |
| 2FI vs Linear | 7.950E-005 | 3 | 2.650E-005 | 3.70 | 0.0504 | Suggested |
| Quadratic vs 2FI | 3.364E-005 | 3 | 1.121E-005 | 2.06 | 0.1937 | – |
| Cubic vs Quadratic | 3.325E-005 | 3 | 1.108E-005 | 9.24 | 0.0286 | Aliased |
| Residual | 4.800E-006 | 4 | 1.200E-006 | – | – | – |
| Total | 12.28 | 17 | 0.72 | – | – | – |

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**Table 5: ANOVA for Response 2 – Tailing Factor (Tf)**

| Source | Sum of Squares | df | Mean Square | F-value | p-value | Remarks |
|---|---|---|---|---|---|---|
| Model | 1.693E-004 | 6 | 2.821E-005 | 3.93 | 0.0278 | Significant |
| A – Buffer pH | 5.513E-005 | 1 | 5.513E-005 | 7.69 | 0.0197 | – |
| B – Organic Ratio | 1.012E-005 | 1 | 1.012E-005 | 1.41 | 0.2621 | – |
| C – Flow Rate | 2.450E-005 | 1 | 2.450E-005 | 3.42 | 0.0943 | – |
| AB | 4.900E-005 | 1 | 4.900E-005 | 6.83 | 0.0258 | – |
| AC | 3.025E-005 | 1 | 3.025E-005 | 4.22 | 0.0670 | – |
| BC | 2.500E-007 | 1 | 2.500E-007 | 0.035 | 0.8556 | – |
| Residual | 7.169E-005 | 10 | 7.169E-006 | – | – | – |
| Lack of Fit | 6.689E-005 | 6 | 1.115E-005 | 9.29 | 0.0246 | Significant |
| Pure Error | 4.800E-006 | 4 | 1.200E-006 | – | – | – |
| Cor Total | 2.409E-004 | 16 | – | – | – | – |

### *3.2. Diagnostic Plots*

Figures 2–11 illustrate the diagnostic plots generated from the BBD analysis for both responses. Normal probability plots of residuals (Figs. 2 and 7) indicate that residuals are approximately normally distributed, confirming model validity. Residuals vs. Run plots (Figs. 3 and 8) show no systematic patterns, indicating model adequacy. Predicted vs. Actual plots (Figs. 4 and 9) demonstrate a strong correlation between experimental and predicted values. Three-dimensional response surface plots (Figs. 5 and 6) depict the influence of critical method parameters on chromatographic responses.

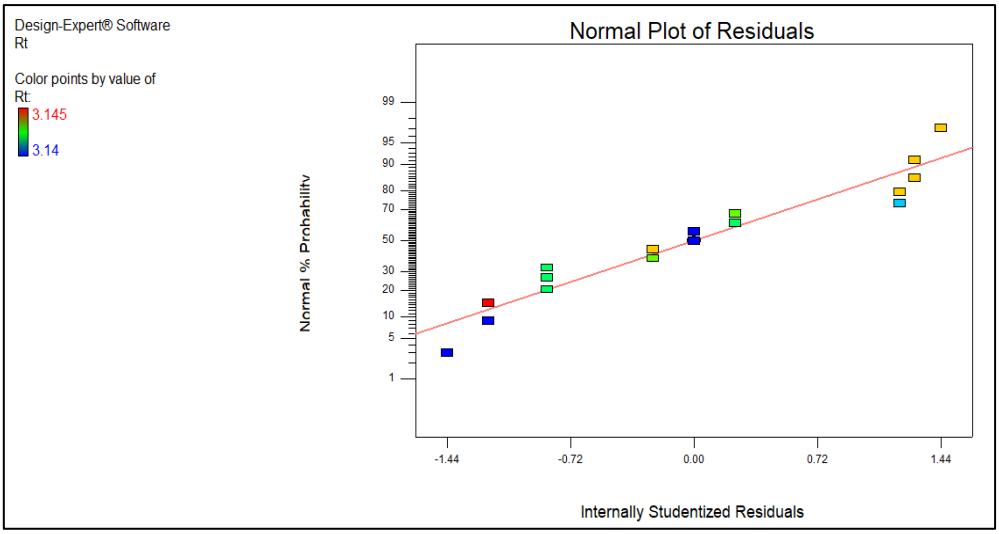



**Fig. 2: Normal Probability Plot of Residuals for Retention Time**

International Journal of Pharmacy and Pharmaceutical Research (IJPPR)
Volume 32, Issue 7, July 2026 ijppr.humanjournals.com ISSN: 2349-7203

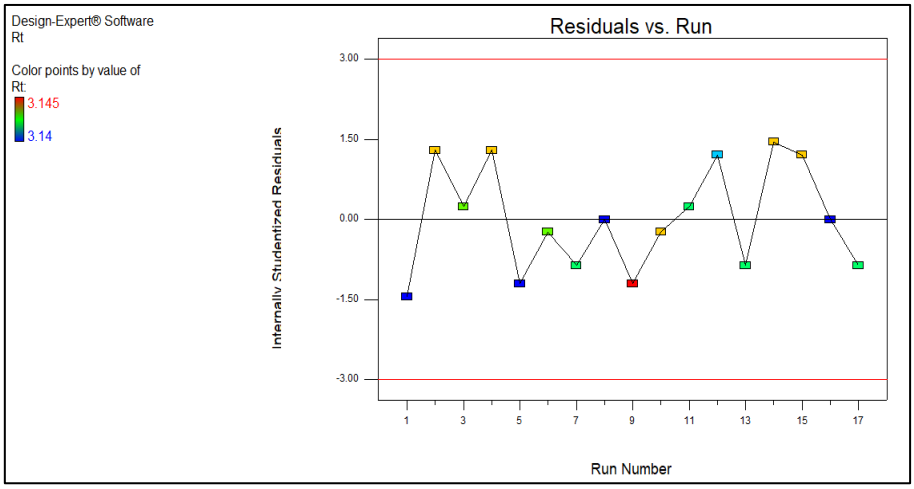



**Fig. 3: Residuals vs. Run for Retention Time**

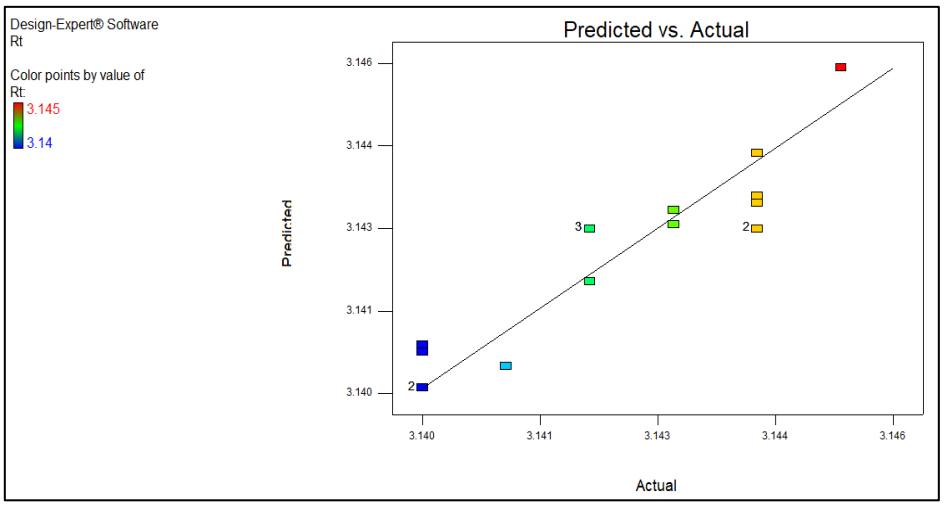



**Fig. 4: Predicted vs. Actual Plot for Retention Time**

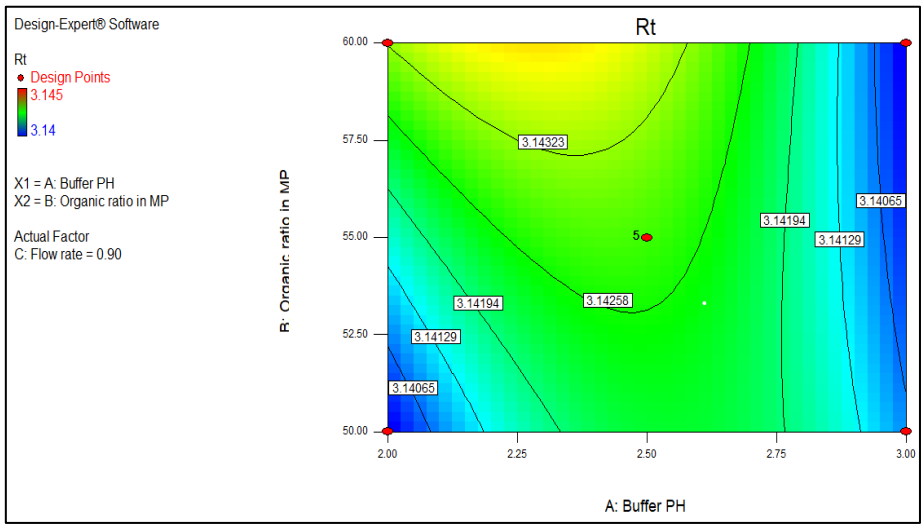



**Fig. 5: Chromatogram Showing Retention Time of Baloxavir Marboxil (Standard)**

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Volume 32, Issue 7, July 2026 ijppr.humanjournals.com ISSN: 2349-7203

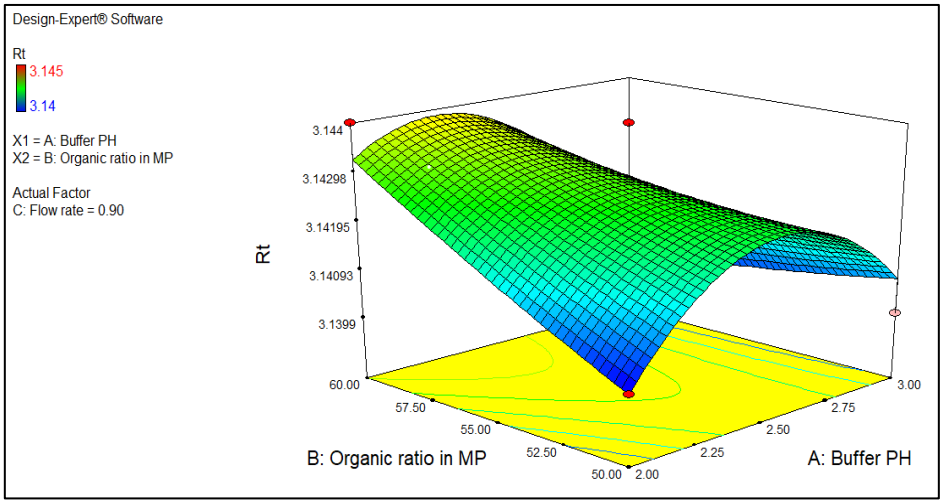



**Fig. 6: 3D Response Surface Plot for Tailing Factor (Buffer pH vs. Flow Rate)**

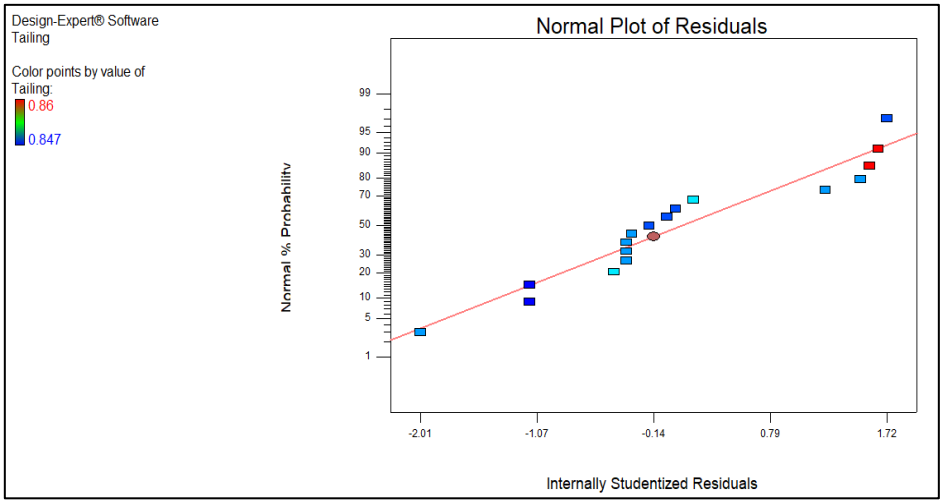



**Fig. 7: Normal Probability Plot of Residuals for Tailing Factor**

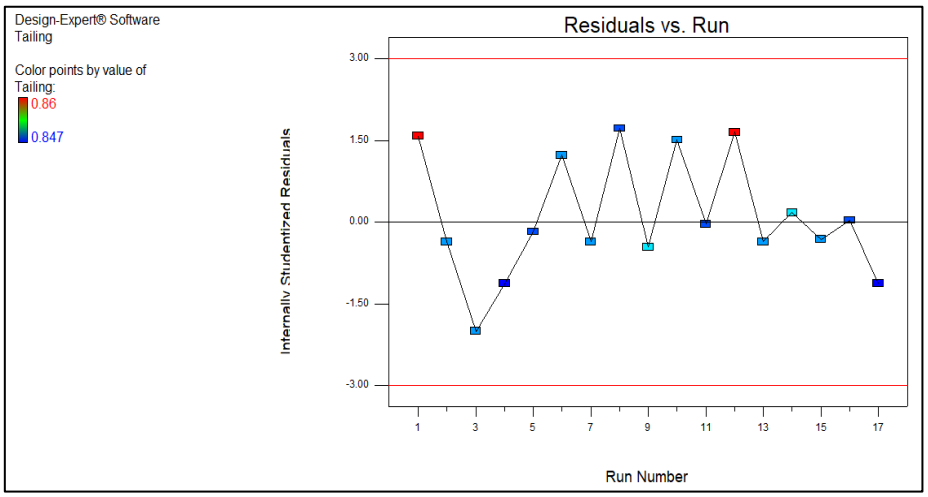



**Fig. 8: Residuals vs. Run for Tailing Factor**

International Journal of Pharmacy and Pharmaceutical Research (IJPPR)
Volume 32, Issue 7, July 2026 ijppr.humanjournals.com ISSN: 2349-7203

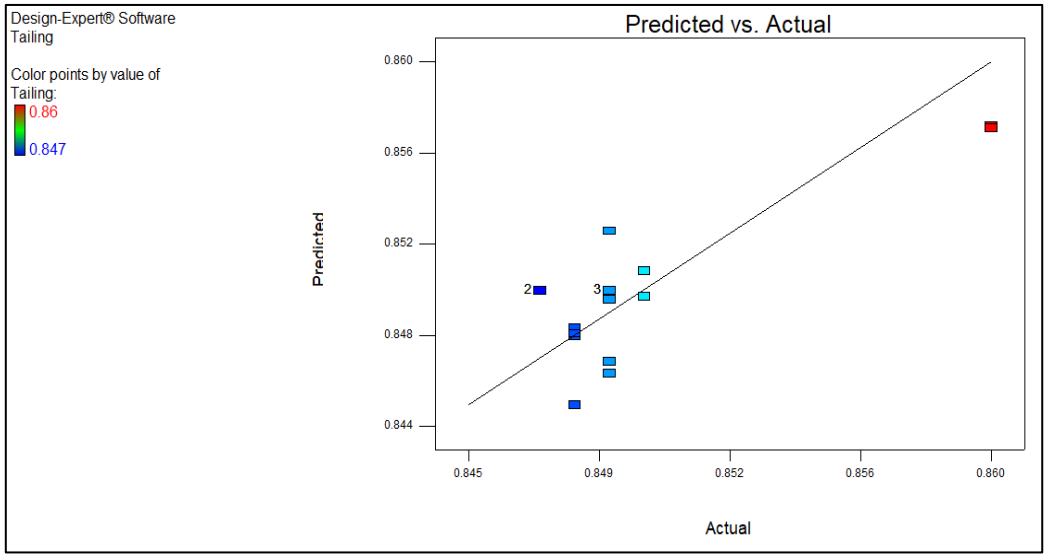



**Fig. 9: Predicted vs. Actual Plot for Tailing Factor**

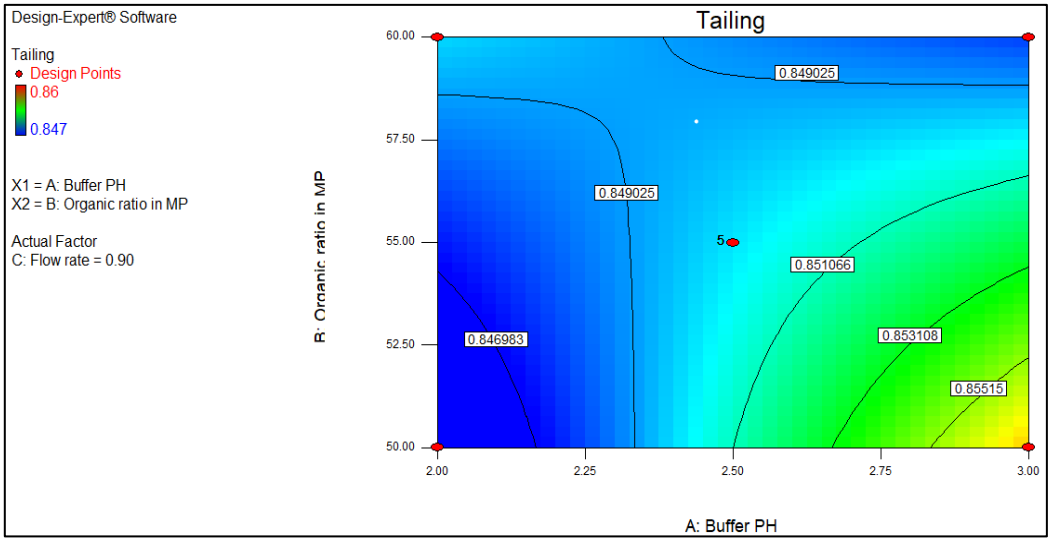



**Fig. 10: 3D Response Surface Plot for Tailing Factor (Buffer pH vs. Organic Ratio)**

### *3.3. System Suitability*

System suitability testing was performed by injecting the standard solution six times under optimized conditions. The mean tailing factor was 1.16 and the mean theoretical plate count was 3330, both satisfying the prescribed acceptance criteria ($Tf \leq 2.0$; $N \geq 2000$).

### *3.4. Validation Results*

Linearity: The method demonstrated excellent linear response over the concentration range of 10–50 µg/mL, with a correlation coefficient ($R^2$) of 0.9998. The regression equation was $y = mx + c$ (where m and c are slope and intercept, respectively).

Precision: Intra-day and inter-day precision yielded %RSD values below 1.0% in all cases, indicating satisfactory repeatability and intermediate precision.

Accuracy: Recovery values ranged from 98.0% to 102.0% at all three spiking levels (80%, 100%, and 120%), confirming the accuracy of the method.

Sensitivity: The LOD and LOQ were determined to be 0.37 ppm and 1.25 ppm, respectively, based on the S/N ratio method (Table 6 and Table 7).

**Table 6: Results of Limit of Detection (LOD) for Baloxavir Marboxil**

| Drug | Baseline Noise (µV) | Signal Obtained (µV) | S/N Ratio | Conc. (ppm) |
|---|---|---|---|---|
| Baloxavir Marboxil | 53 | 155 | 2.92 | 0.37 |

**Table 7: Results of Limit of Quantitation (LOQ) for Baloxavir Marboxil**

| Drug | Baseline Noise (µV) | Signal Obtained (µV) | S/N Ratio | Conc. (ppm) |
|---|---|---|---|---|
| Baloxavir Marboxil | 53 | 527 | 9.94 | 1.25 |

## 4. DISCUSSION

The application of Quality by Design to RP-HPLC method development represents a systematic, knowledge-based approach that addresses the limitations of traditional trial-and-error optimization. In this study, Box–Behnken Design was employed to evaluate the effects of buffer pH, organic phase ratio, and flow rate on the chromatographic behavior of baloxavir marboxil. The choice of a 3-factor, 3-level BBD was driven by its efficiency in fitting a second-order response surface model with a smaller number of runs compared to a full factorial or central composite design, while still providing sufficient information on curvature and interactions [7].

ANOVA of the BBD results revealed that buffer pH (A) was the most statistically significant factor influencing the tailing factor ($p = 0.0197$), consistent with the role of pH in modulating ionization states of the analyte and silanophilic interactions with the stationary phase. The interaction term AB (buffer pH × organic ratio) also reached statistical significance ($p = 0.0258$), indicating that these two parameters should be optimized together rather than independently. The flow rate and its interaction terms showed a lesser but notable influence on tailing, which has practical implications for robustness.

The optimized conditions ($KH_2PO_4$ buffer pH 4.5, methanol:buffer 60:40 v/v, flow rate 1.0 mL/min, PLATISIL C18 column) yielded a well-resolved peak with a tailing factor of 1.16 and theoretical plate count of 3330, both well within ICH-recommended limits. These values are comparable to those reported by Divya and Pawar (2022) [10], who developed an RP-HPLC–PDA method for BXM and its impurities using an Inertsil ODS-3V column, albeit without QbD optimization.

The linearity range of 10–50 µg/mL with $R^2 = 0.9998$ is consistent with previously published RP-HPLC methods for BXM. Nagulancha et al. (2023) [8] reported a stability-indicating RP-HPLC method with linearity in the range of 5–150 µg/mL; however, the incorporation of QbD in the present study provides a more scientifically justified design space and enhances regulatory acceptability. Gouda et al. (2023) [12] reported a UHPLC–PDA method with comparable sensitivity parameters.

Precision results (%RSD < 1%) are in agreement with ICH Q2(R1) requirements and compare favourably with existing literature. Recovery values between 98 and 102% confirm that tablet excipients do not interfere with the determination of BXM. The LOD (0.37 ppm) and LOQ (1.25 ppm) values indicate adequate sensitivity for trace-level detection, which is relevant for stability studies and impurity profiling, as emphasized by Hafez et al. (2022) [13].

The robustness assessment demonstrated that deliberate variations in chromatographic conditions did not significantly affect the system suitability parameters, confirming that the method operates within a defined design space. This is a key advantage of QbD-based method development, as it ensures that small changes encountered during routine analysis do not compromise method performance [6].

Overall, the present QbD-assisted RP-HPLC method offers several advantages over previously reported methods: (i) systematic optimization using response surface methodology, (ii) comprehensive statistical evaluation of factor effects and interactions, (iii) full ICH Q2(R1) compliance, and (iv) suitability for routine quality control and regulatory submission.

## 5. CONCLUSION

A robust, sensitive, and fully validated RP-HPLC method was successfully developed for the quantitative estimation of baloxavir marboxil in bulk drug and pharmaceutical tablet dosage forms. The integration of a Quality by Design approach using Box–Behnken Design allowed systematic optimization of critical method parameters—buffer pH, organic phase ratio, and flow rate—and provided a well-defined design space ensuring consistent chromatographic performance. The validated method complied with all ICH Q2(R1) criteria for linearity ($R^2$ = 0.9998; 10–50 µg/mL), precision (%RSD < 1%), accuracy (98–102% recovery), and sensitivity (LOD: 0.37 ppm; LOQ: 1.25 ppm). System suitability parameters were consistently within acceptance limits. The method is specific, reproducible, rugged, and robust, rendering it applicable for routine quality control, stability testing, and regulatory analysis of baloxavir marboxil formulations in pharmaceutical manufacturing environments.

## ETHICAL APPROVAL STATEMENT

The present study involved only in vitro analytical experimentation using bulk drug substance and pharmaceutical dosage forms. No human subjects, animals, or biological specimens were utilized. Therefore, formal ethics committee approval was not required for this investigation. However, authors are advised to include an appropriate ethics statement if any human or animal data are incorporated in the final submission.

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