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Quality by Design Approach in HPLC Method Development and Validation of Baloxavir Marboxil in Pharmaceutical Dosage Form

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A B S T R A C T

A simple, robust, and validated RP-HPLC method was developed for the quantitative estimation of Baloxavir in bulk and pharmaceutical dosage forms, as per ICH Q2(R1) guidelines. Chromatographic separation was achieved on a PLATISIL C18 column (4.6×250 mm, 5 µm) using a mobile phase of methanol and KH2PO4 buffer (pH 4.5) in a 60:40 v/v ratio, at a flow rate of 1 mL/min, with UV detection at 247 nm. System suitability parameters, including theoretical plates (>2000) and tailing factor (<2), complied with acceptance criteria, confirming method reliability. The method exhibited excellent linearity in the range of 10–50 µg/mL with a correlation coefficient (R^2) of 0.9998. Precision studies yielded %RSD < 1%, while accuracy tests showed recoveries within 98–102%. Sensitivity studies revealed a LOD of 0.37 ppm and LOQ of 1.25 ppm, indicating high detection capability. Robustness and ruggedness evaluations confirmed the stability of the method under small, deliberate changes in chromatographic conditions. Overall, the validated RP-HPLC method is specific, precise, accurate, and reproducible**,** making it highly suitable for routine quality control, stability assessment, and regulatory analysis of Baloxavir formulations in the pharmaceutical industry.

Keywords: Baloxavir, RP-HPLC, Method Validation, ICH Q2(R1), Robustness, Quality Control.

A R T I C L E I N F O

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C O N T E N T S

1. Introduction

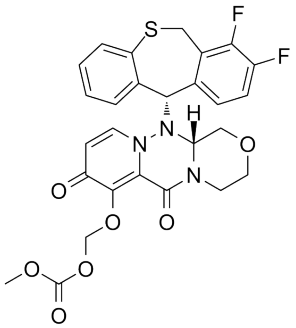

Fig.1: Baloxavir Marboxil

Table 1: Drug profile

Molecular Formula	$C_{20}H_{28}N_2O_5S$
Molecular Weight	408.52 g/mol
IUPAC Name	5-[(2R)-2-[[2-(2-Ethoxyphenoxy) ethyl]amino]propyl]-2-methoxybenzenesulfonamide
Chem Spider ID	4444067
Density	1.22 g/cm3
Boiling Point	543.4°C

Vapour Pressure	1.43E-10 mmHg
Flash Point	233.8°C
Refractive Index	1.54
Polar Surface Area	84.36 Å2
LogP (Octanol/Water)	3.3
Generic Name	Baloxavir Marboxil
Brand Names	Flomax, Urimax
Drug category	Alpha-1 adrenergic receptor antagonist
Indications	Benign Prostatic Hyperplasia (BPH
Pharmacology	Relaxation of smooth muscle in the prostate and bladder neck
Potency	High affinity for alpha-1A and alpha-1D adrenergic receptors
Tolerability	Generally well-tolerated, but may cause orthostatic hypotension and dizziness
Contraindications	Hypersensitivity to or any component of the formulation |
Adverse Effects	Orthostatic hypotension, dizziness, headache, fatigue, nasal congestion
Availability	Prescription-only medication, available in oral capsules or tablets
Mechanism of Action	Baloxavir Marboxil works by selectively blocking alpha-1 adrenergic receptors in the prostate and bladder neck, leading to relaxation of smooth muscle in these areas and improved urine flow

2. Materials and Methods

Table 2: Instruments used

S.No	Instrument	Model
1	HPLC	WATERS, software: Empower, 2695 separation module.2487 UV detector.
2	UV/VIS spectrophotometer	LABINDIA UV 3000$^+$
3	pH meter	Adwa – AD 1020
4	Weighing machine	Afcoset ER-200A
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil

Table 3: Chemicals used

S.No	Chemical	Brand
1	Baloxavir	Supplied by MSN LAB
2	KH_2PO_4	FINAR chemical LTD
3	Water and Methanol for HPLC	Standard solutions Ltd
4	Acetonitrile for HPLC	Standard solutions Ltd
5	HCl, H_2O_2, NaOH	MERCK

Optimization of Column:

PLATISIL C18 (4.6 x 250mm, 5µm) was found to be ideal as it gave good peak shape and resolution at 1.0 ml/min flow.

Optimized chromatographic conditions:

Instrument used : High performance liquid chromatography equipped with Auto Sampler and PDA detector

Temperature : Ambient

Column : Platisil C18 (4.6 x 250mm, 5µm)

Mobile phase: Methanol: KH2PO4 PH 4.5 (60: 40v/v)

Flow rate : 1ml/min

Wavelength : 247 nm

Injection volume : 20 µl

Run time : 10 min.

Preparation of Buffer and Mobile Phase:

Preparation of KH2PO4 pH 4.5:

To prepare phosphate buffer solution, by adding 0.1ml of formic acid in a 1000ml water. Adjust this solution to pH 4.5 by using sodium hydroxide.

Preparation of mobile phase:

Mix a mixture of above buffer 400ml (40%), 600 ml Methanol (60%) and degas in ultrasonic water bath for 5 minutes. Filter through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

Methanol: KH2PO4 PH 4.5 (60:40) ratio.

System Suitability:

Tailing factor for the peaks due to Baloxavir in Standard solution should not be more than 2.0

Theoretical plates for the Baloxavir peaks in Standard solution should not be less than 2000

Calculation: (For Baloxavir)

Calculation: (For Ganaxolone)

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

Where:

AT = average area counts of sample preparation.

AS = average area counts of standard preparation.

WS= Weight of working standard taken in mg.

P= Percentage purity of working standard

LC= Label Claim mg/ml.

System Suitability Results:

1. Tailing factor Obtained from the standard injection is 1.16
2. Theoretical Plates Obtained from the standard injection is 333

Validation Parameters

Assay:

Standard Solution Preparation: Accurately weigh and transfer 25 mg of Baloxavir working standard into a two 25 ml clean dry volumetric flasks add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3ml of each of the above stock solutions into a two 10ml volumetric flasks and dilute up to the mark with Diluents.

Sample Solution Preparation:

Accurately weigh and transfer equivalent to 25 mg of Baloxavir equivalent weight of the sample into a two 25 ml clean dry volumetric flasks add about 7ml of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of each of the above stock solution into a two 10ml volumetric flasks and dilute up to the mark with Diluents.

Procedure: Inject 10 µL of the standard, sample into the chromatographic system and measure the areas for the Baloxavir peaks and calculate the % Assay by using the formulae.

Linearity:

Preparation of stock solution:

Accurately weigh and transfer 25 mg of Baloxavir working standard into a two 25 ml clean dry volumetric flasks add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (10ppm of Baloxavir)

0.1ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – II (20ppm of Baloxavir):

0.2ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – III (30ppm of Baloxavir):

0.3ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – IV (40ppm of Baloxavir):

0.4ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Preparation of Level – V (50ppm of Baloxavir):

0.5ml of stock solution has taken in 10ml of volumetric flask dilute up to the mark with Diluents.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

Precision:

Preparation of stock Solution:

Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Procedure: The standard solution was injected for Six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Intermediate precision/ruggedness:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different day within the laboratory.

Preparation of stock solution: Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

For accuracy determination, three different concentrations were prepared separately i.e. 50%, 100% and 150% for the analyte and chromatograms are recorded for the same.

Preparation of Standard stock solution:

Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)**.** Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Preparation Sample solutions:

For preparation of 50% solution (With respect to target Assay concentration): Accurately weigh and transfer 12.5mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

For preparation of 100% solution (With respect to target Assay concentration): Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

For preparation of 150% solution (With respect to target Assay concentration): Accurately weigh and transfer 37.5 mg of Baloxavir working standard into a 25ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents.

Procedure:

Inject the standard solution, Accuracy -50%, Accuracy -100% and Accuracy -150% solutions. Calculate the Amount found and Amount added for Baloxavir and calculate the individual recovery and mean recovery values.

Limit of detection:

Preparation of Baloxavir solution:

Preparation of 0.37 µg/ml solution:

Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution

into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1.24 ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.(0.4ppm).

Limit of quantification:

Preparation of Baloxavir solution:

Preparation of 1.25µg/ml solution: Accurately weigh and transfer 25 mg of Baloxavir working standard into a 25 ml clean dry volumetric flask add Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipette 0.3ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 1ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluents. Further pipette 4.18ml of the above stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent (1.3ppm).

Robustness: As part of the Robustness, deliberate change in the Flow rate, Mobile Phase composition, Temperature Variation was made to evaluate the impact on the method.

The flow rate was varied at 0.8 ml/min to 1.2 ml/min: Standard solution 30 µg/ml of Baloxavir prepared and analyzed using the varied flow rates along with method flow rate.

The Organic composition in the Mobile phase was varied from 54% to 66%: Standard solution 30µg/ml of Baloxavir was prepared and analyzed using the varied Mobile phase composition along with the actual mobile phase composition in the method.

Table 4: Development by QBD Optimization

		Factor 1	Factor 2	Factor 3	Response 2	Response 3
Run		A: Buffer pH	B: Mobile Phase Ratio	C: Flow Rate	Retention Time	Tailing Factor
2	1	3.00	50.00	0.90	3.14	0.86
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	3.14	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	3.14	0.848
12	9	2.50	60.00	1.00	3.145	0.85
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	0.86
15	13	2.50	55.00	0.90	3.142	0.849
3	14	2.00	60.00	0.90	3.144	0.85
9	15	2.50	50.00	0.80	3.144	0.849
4	16	3.00	60.00	0.90	3.14	0.848
14	17	2.50	55.00	0.90	3.142	0.847

Table 5: Software Information

File Version	22.0.4.0		
Study Type	Response Surface	**Subtype**	Randomized
Design Type	Box-Behnken	**Runs**	17
Design Model	Quadratic	**Blocks**	No Blocks
Build Time (ms)	4.00		

Table 6: Factors

Factor	Name	Units	Type	Low actual	high actual	Low coded	High coded	mean	Std dev
A	Buffer pH		Numeric	2.00	3.00	-1.000	1.000	2.500	0.343
B	Mobile Phase Ratio		Numeric	50.00	60.00	-1.000	1.000	55.000	3.430
C	Flow Rate		Numeric	0.80	1.00	-1.000	1.000	0.900	0.069

Table 7: Responses

Response	Name	Units	Obs	Analysis	Minimum	Maximum	Mean	Std. Dev.	Ratio	Trans	Model
Y1	Rt	min	17	Polynomial	3.14	3.15	3.14	1.643E-003	1.00	None	Quadratic
Y2	Tailing		17	Polynomial	0.85	0.86	0.85	3.765E-003	1.02	None	No model chosen

3. Results and Discussion

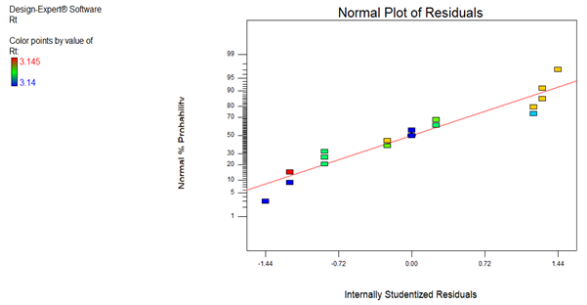

Fig.2: Standard Calibration graph values of Mirtazapine in 0.1N HCL

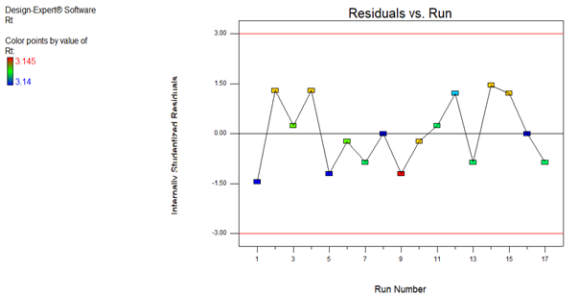

Fig.3: Residuals vs. Run for Baloxavir

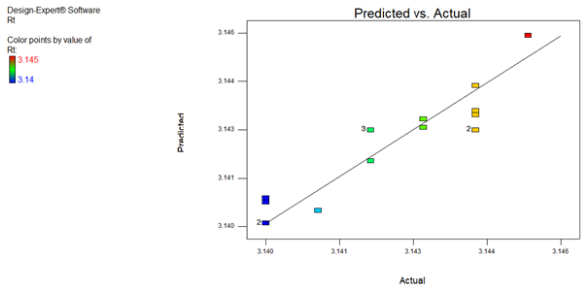

Fig.4: Predicted vs. Actual for Baloxavir

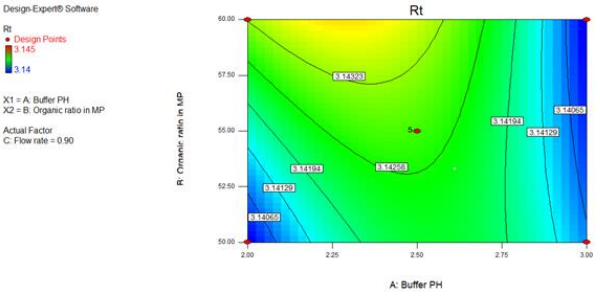

Fig.5: Retention time for Baloxavir

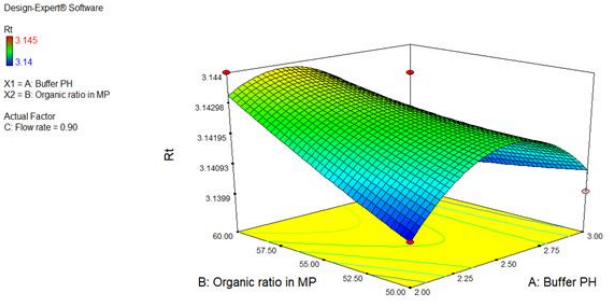

Fig.6: 3D Surface for Baloxavir

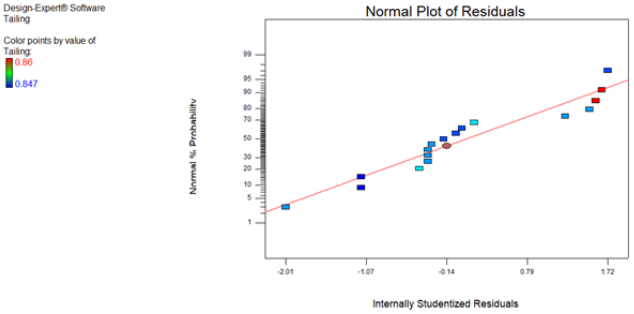

Fig.7: Normal plot of Residuals for Baloxavir

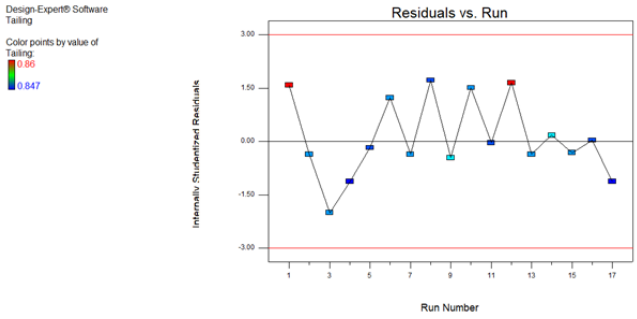

Fig.8: Residulas vs.Run for Baloxavir

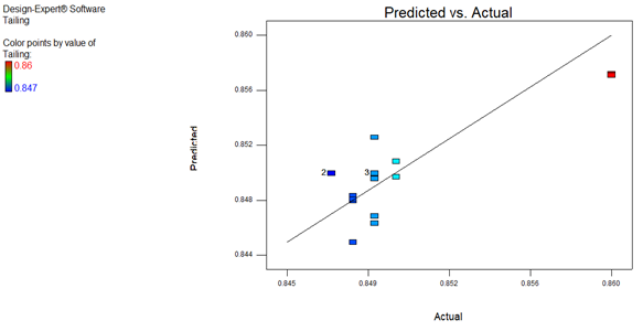

Fig.9: Predicted vs.Actial for Baloxavir

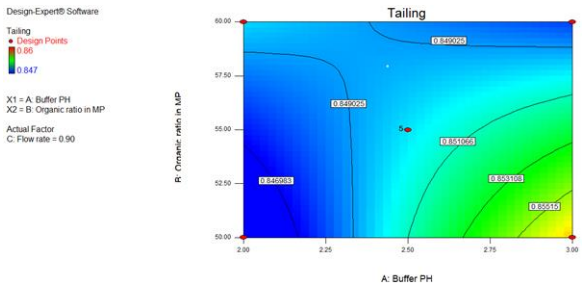

Fig.10: R tailing factor for Baloxavir

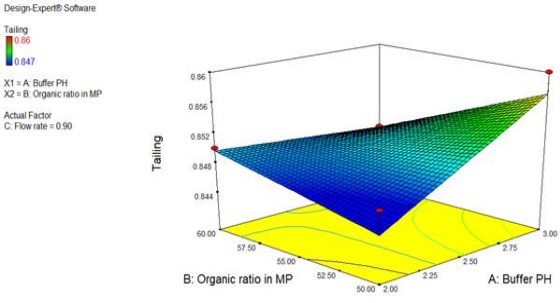

Fig.11: 3D Surface for Baloxavir

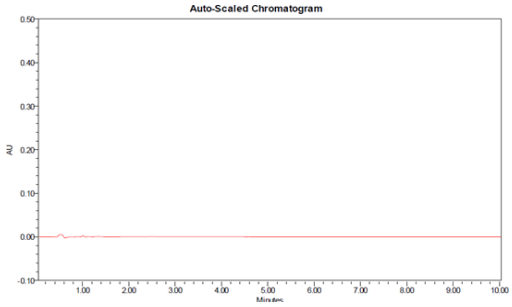

Fig.12: Chromatogram for system suitability

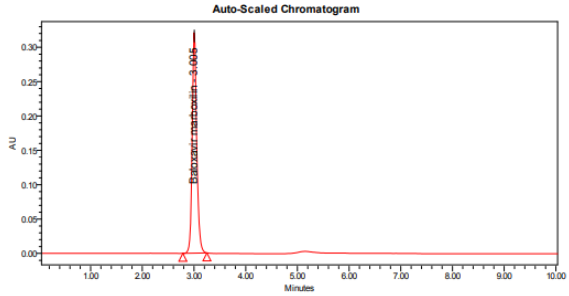

Fig.13: Chromatogram for system suitability of Standard solution

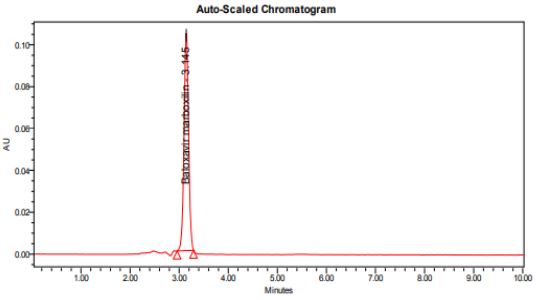

Fig.14: Chromatogram for system suitability of Sample solution

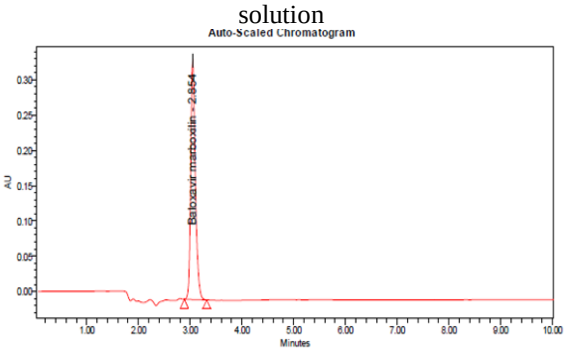

Fig.15: Chromatogram for Standard

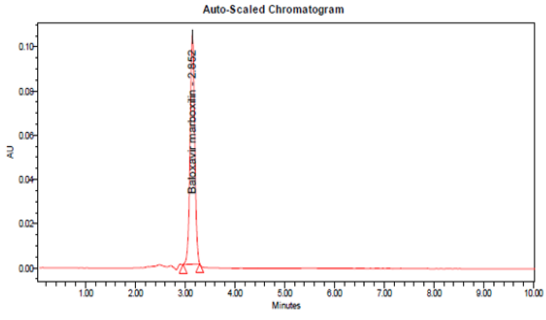

Fig.16: Chromatogram for Sample

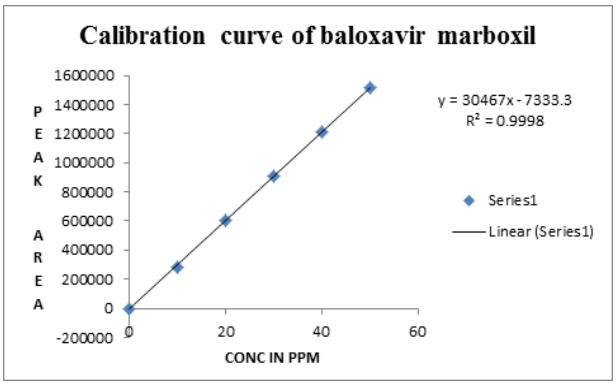

Fig.17: Analytical performance parameters of Baloxavir

Table 12: Area of different concentration of Baloxavir

S.No	**Concentration (µg/ml)**	**Areas of Baloxavir**
1	10	283072
2	20	607144
3	30	908216
4	40	1213288
5	50	1514360

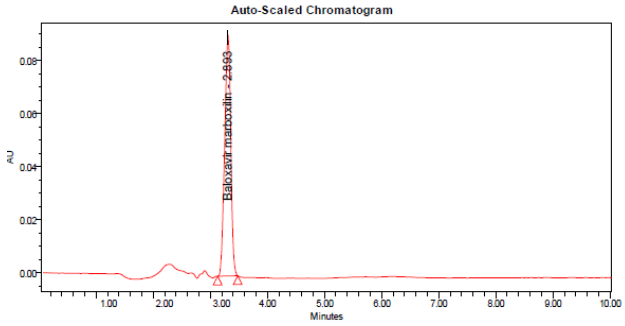

Fig.18: Chromatogram for Precision -6

Table 8: Results of Precision for Baloxavir

Injection	**Area**
Injection-1	917456
Injection-2	918584
Injection-3	919369
Injection-4	912147
Injection-5	928210
Injection-6	919241
Average	919167.8
Standard Deviation	5184.013
%RSD	0.6

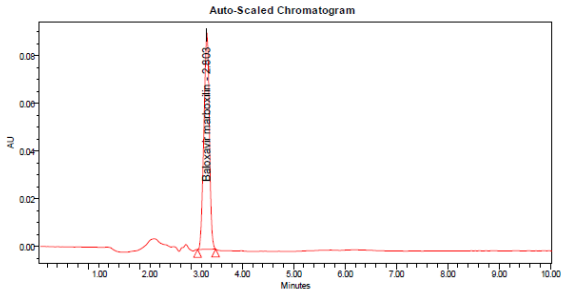

Fig.19: Chromatogram for ID Precision -6

Table 9: Results of Intermediate precision for Baloxavir

Injection	Area
Injection-1	929586
Injection-2	928254
Injection-3	923963
Injection-4	921475
Injection-5	917258
Injection-6	929874
Average	925068.3
Standard Deviation	5075.181
%RSD	0.5

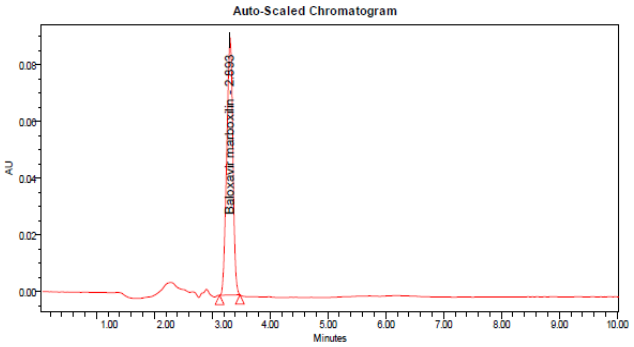

Fig.20: Chromatogram for Accuracy 50%-3

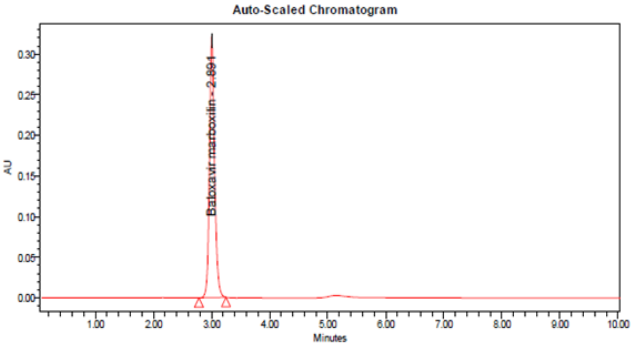

Fig.21: Chromatogram for Accuracy 100%-3

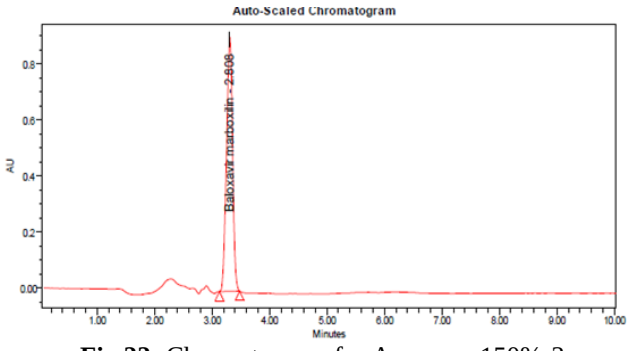

Fig.22: Chromatogram for Accuracy 150%-3

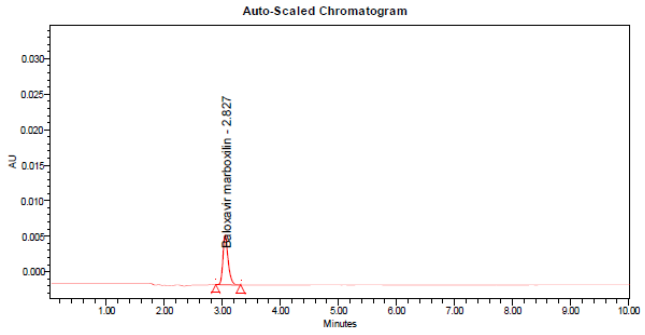

Fig.23: Chromatogram of Baloxavir showing LOD

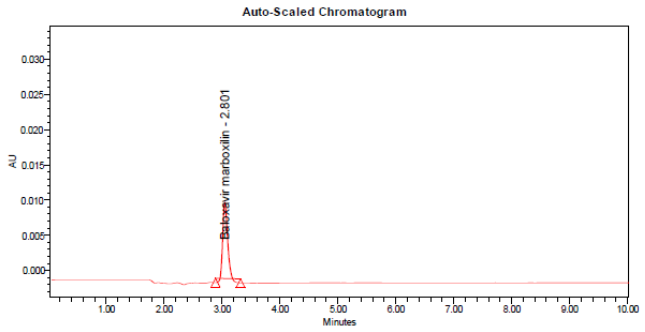

Fig.24: Chromatogram of Baloxavir showing LOQ

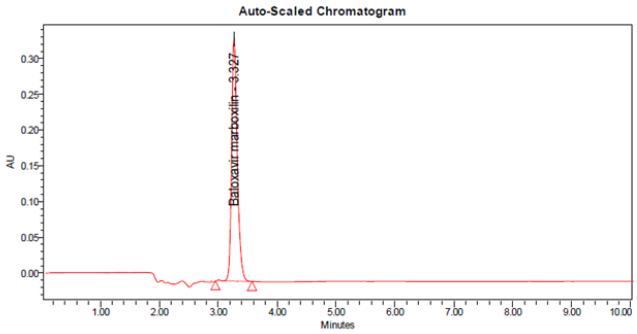

Fig.25: Chromatogram showing less organic composition

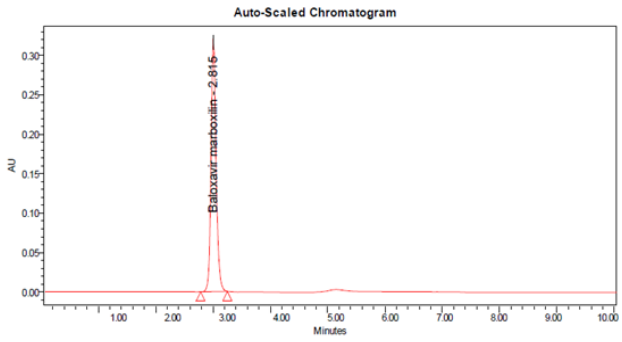

Fig.26: Chromatogram showing more organic composition

Table 10: Results for variation in flow for Baloxavir

S. No	Flow Rate (ml/min)	System Suitability Results	
		USP Plate Count	USP Tailing
1	0.8	3584	1.3
2	1	3641	1.2
3	1.2	3794	1.5

Table 11: Results for variation in mobile phase composition for Baloxavir

S.No	Change in OrganicComposition in theMobile Phase	System Suitability Results	
		USP Plate Count	USP Tailing
1	10% less	3245	0.86
2	*Actual	3212	0.85
3	10% more	3239	0.84

Table 12: Accuracy (recovery) data for Baloxavir

%Concentration (at specification Level)	Area*	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	450123	12.5	**12.36**	98.89	**99.2**
100	901245	25	**24.90**	99.6	
150	1351368	37.5	**37.11**	99.0	

Table 13: FIT Summary

Source	Sum of Squares	df	Mean Square	F-Value	P value Prob > F	
Mean vs Total	167.86	1	167.86			Suggested
Linear vs Mean	8.250E-006	3	2.750E-006	0.95	0.4451	
2FI vs Linear	1.250E-005	3	4.167E-006	1.66	0.2383	
Quadratic vs 2FI	1.758E-005	3	5.861E-006	5.43	0.0303	Suggested
Cubic vs Quadratic	2.750E-006	3	9.167E-007	0.76	0.5705	Aliased
Residual	4.800E-006	4	1.200E-006			Suggested
Total	167.86	17	9.87			

ANOVA for Quadratic model: Table 14: Response 1: Retention time

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.833E-005	9	4.259E-006	3.95	0.0419	significant
A-Buffer PH	3.125E-006	1	3.125E-006	2.90	0.1325	
B-Organic ratio in MP	3.125E-006	1	3.125E-006	2.90	0.1325	
C-Flow rate	2.000E-006	1	2.000E-006	1.85	0.2155	
AB	4.000E-006	1	4.000E-006	3.71	0.0955	
AC	6.250E-006	1	6.250E-006	5.79	0.0470	
BC	2.250E-006	1	2.250E-006	2.09	0.1919	
A^2	1.520E-005	1	1.520E-005	14.09	0.0071	
B^2	4.211E-008	1	4.211E-008	0.039	0.8490	
C^2	3.042E-006	1	3.042E-006	2.82	0.1370	
Residual	7.550E-006	7	1.079E-006			
Lack of Fit	2.750E-006	3	9.167E-007	0.76	0.5705	
Pure Error	4.800E-006	4	1.200E-006			
Cor Total	4.588E-005	16				

Table 15: Response 2: tailing factor

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	12.28	1	12.28			Suggested
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			

Source	Sum of Squares	df	Mean Square	F-value	p-value	
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 in MP	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		0.0278	
Cor Total	2.409E-004	16	2.821E-005			

Table 16: Results of LOD

Drug name	Baseline noise(µV)	Signal obtained(µV)	S/N ratio	Conc. In ppm
Baloxavir	53	155	2.92	0.37

Table 17: Results of LOQ

Drug name	Baseline noise(µV)	Signal obtained (µV)	S/N ratio	Conc. In ppm
Baloxavir	53	527	9.94	1.25

4. Conclusion

A robust and validated RP-HPLC method was successfully developed for the estimation of Baloxavir in bulk and pharmaceutical dosage forms, in accordance with ICH Q2(R1) guidelines. Optimization using a quadratic design confirmed that buffer pH, organic ratio, and flow rate significantly influenced retention time and tailing factor. The chromatographic separation was achieved using a PLATISIL C18 column (4.6 × 250mm, 5µm) with a mobile phase of methanol and KH2PO4 buffer (pH 4.5) in a 60:40 ratio, at a flow rate of 1 mL/min, and detection wavelength of 247 nm. System suitability results met all acceptance limits, with theoretical plates greater than 2000 and tailing factor less than 2. Validation studies showed excellent linearity in the range of 10–50 µg/mL (R^2 = 0.9998), precision with %RSD < 1%, and accuracy within the recovery range of 98–102%. The method also demonstrated good sensitivity with LOD (0.37 ppm) and LOQ (1.25 ppm), confirming its suitability for detecting low concentrations. Robustness and ruggedness studies confirmed the method's reliability under deliberate variations in flow rate and mobile phase composition, with no significant impact on system suitability parameters. Overall, the developed RP-HPLC method is simple, precise, accurate, specific, and reproducible, making it highly applicable for routine quality control, stability testing, and regulatory submissions of Baloxavir formulations. Its efficiency, short run time, and compliance with international guidelines make it a dependable analytical tool for pharmaceutical industries to ensure consistent quality and therapeutic performance of Baloxavir-based products.

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