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Development of RP-HPLC PDA method for concurrent quantification of Baloxavir marboxil and its impurities

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Abstract: For the quantification of contaminants in Baloxavir marboxil (BXML), a simple and accurate RP-HPLC method was established and validated. BXML is an antiviral agent used to treat infections caused by influenza A and B viruses. To achieve optimal separation of all five impurities along with main moiety BXML the chromatography was carried out with X-Bridge Phenyl (150 x 4.6 mm) 3.5µ column with a mobile phase flow speed of 0.5 mL/minute. The mobile phase comprised of methanol and KH_2PO_4 buffer (pH 2.5) in gradient mode. The retention periods of Imp - 1, Imp - 2, Baloxavir marboxil, Imp - 3, Imp - 4 and Imp - 5 were found to be 1.919, 3.264, 4.429, 7.053, 8.247 and 8.937 min respectively. The developed method was found to be specific and linear ($R^2 > 0.999$) for quantification of the target analytes. LOD was established as 0.020, 0.002, 0.001, 0.005, 0.002 and 0.002 µg/mL for BXML, Impurities 1, 2, 3, 4 and 5 respectively. Similarly, the LOQ was established as 0.066, 0.006, 0.003, 0.016, 0.006 and 0.006 µg/mL for Baloxavir marboxil, its Impurities 1,2,3,4 and 5 respectively. The % recovery by the assay was determined within the range of 98 –102%.

Keywords: Baloxavir marboxil; method development; validation; impurity; RP-HPLC.

1. Introduction

In the present scenario, a multiple number of entities have entered into pharmaceutical manufacturing, research and development business with numerous drugs. The development of every single drug is diverse from each other. Various kinds of drug synthesis will produce different impurity profiles liable with the process. The resultant impurities may cause adverse effects on human health when they are exposed from time to time. Hence these impurities need to be accurately classified and controlled. Analytical research and development play an important part in pharmaceutical product development and manufacture. Scientifically developed analytical testing approaches deserve the quality, efficacy and adverse effects of the drug product as well as the active pharmaceutical ingredients (API). In the present study drug Baloxavir marboxil [1] selected was highly complicated and quite challenging due to chemical structure and properties. Author aimed to separate the maximum number of impurities which can be potential degradants and possible process related impurities of all the probable synthetic routes. Baloxavir marboxil was recognized by the United States FDA in 2018[2].

Baloxavir marboxil (Figure 1) is [(3R)-2-[(11S)-7,8-difluoro-6,11dihydrobenzo[c][1] benzothiepin-11yl]-9,12-dioxo-5-oxa-1,2,8-triazatricyclo [8.4.0.03,8] tetradeca-10,13-dien-11-yl]

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oxymethyl methyl carbonate [3] and a selective inhibitor of influenza cap-dependent endonuclease which inhibits polymerase function and consequently replication of influenza virus mRNA. It's an antiviral drug that is used to treat influenza A and influenza B infections [4]. It has antiviral activity against influenza A and B viruses, including strains that are resistant to currently available antivirals [5-7]. BXML obstructs the enzymes crucial for replication of viruses and thus acts quickly in treating flu virus infections and also in treatment of symptoms improved related with infection [8].

Table 1. List of related impurities of Baloxavir marboxil

No	Impurity (Imp) Name	Impurity Code
1	7,8-Difluoro-6,11-dihydrodibenzo[b,e]thiepin-11-ol	Imp – 1
2	3-(Benzyloxy)-4-oxo-4h-pyran-2-carboxylic acid	Imp – 2
3	Tert-butyl 2-(3-(benzyloxy)-4-oxo-4H-pyran-2-carbonyl) hydrazine-1-carboxylate	Imp – 3
4	7-fluorodibenzo [b, e] thiepin-11(6H)-one	Imp – 4
5	(R)-7-(benzyloxy)-12-((R)-tetrahydrofuran-2-carbonyl)-3,4,12,12a- tetrahydro-1H-[1,4]oxazino[3,4-c]pyrido[2,1-f][1,2,4]triazine-6,8-dione	Imp – 5

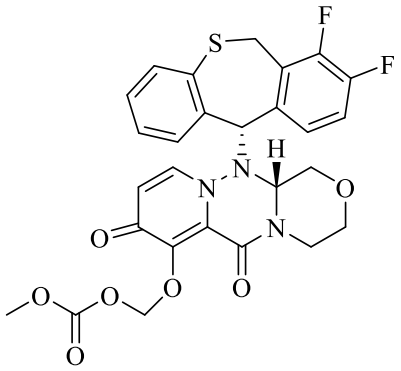

Figure 1. Chemical structure of Baloxavir marboxil

Literature discloses that limited methods are reported for the estimation of Baloxavir marboxil (BXML) by using chromatographic methods which include two bioanalytical LC-MS methods for determination of Baloxavir marboxil [9, 10] and an RP-UPLC technique for the estimation of Baloxavir marboxil in commercial dosage form [11].

From the reported methods it was concluded that no RP-HPLC method was reported till date for the separation and identification of Baloxavir marboxil along with its known impurities. Hence author made a successful attempt to develop rapid, simple RP- HPLC method for quantification of Baloxavir marboxil with five impurities in a single run and validated in accordance with ICH guidelines (ICH Q1A(R2), Q2(R1) and Q3B R2) [12 -14].

Present investigation describes the quantification of five known related substances of Baloxavir marboxil as specified in Table 1. The chemical structure of these related impurities [15-19] were depicted in Figure 2.

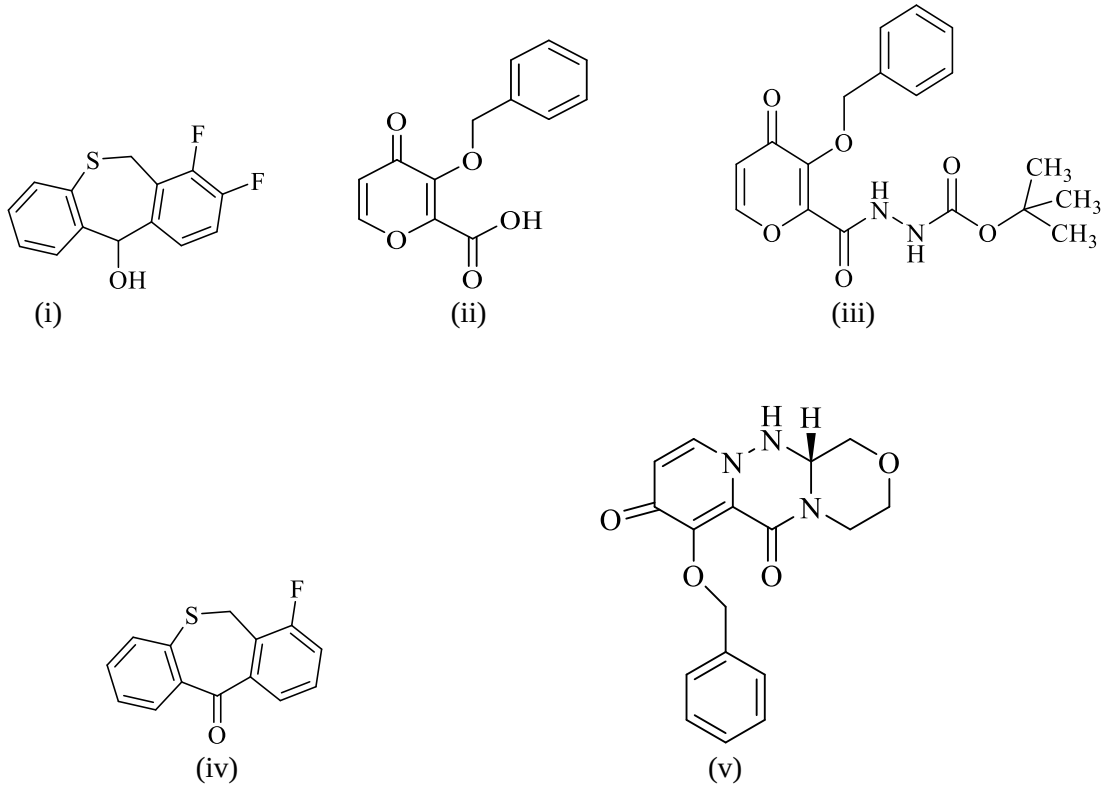

Figure 2. Chemical structure of Baloxavir marboxil impurities.
(i) Imp -1 (ii) Imp - 2 (iii) Imp - 3 (iv) Imp - 4 (v) Imp - 5.

2. Materials and Methods

2.1. Chemicals

Baloxavir marboxil (99.8% purity) and reference standards of 5 impurities (Imp-1 of 99.6%, Imp-2 of 99.9%, Imp- 3 of 99.6 %, Imp-4 of 99.9% and Imp-5 of 99.8% purity) were provided as gift samples from Zydus Cadila, Ahmedabad, India. HPLC grade solvent acetonitrile was purchased from E. Merck, Mumbai, India. Analytical grade chemicals include potassium dihydrogen ortho-phosphate, triethylamine and ortho-phosphoric acid were acquired from Rankem, Avantor performance material India limited.

2.2. Equipment

A Waters Alliance HPLC 2695 separation module furnished with a binary pump, photodiode array detector, and auto sampler. The chromatographic information was attained and handled by Empower-2 software. Denver electronic balance was used for weighing the materials. Digital pH meter 7007 (Digisun Electronics Hyderabad) was used for all pH measurements. Ultrasonic bath (Shiva Scientific technologies) was used for sonication of the samples. Vacuum pump (Crompton), Hot air oven (Servewell Instrument PVT LTD, Bangalore) was used.

2.3. Optimized Chromatographic Settings

The chromatograms were obtained using Empower® version 2 and a Waters Alliance-HPLC system equipped with a 2695-separation module integrated to a 2996-photo diode array detector. The peaks were separated using X-Bridge Phenyl (150 x 4.6 mm) 3.5 micron column with a mobile phase of methanol and KH_2PO_4 buffer (pH 2.5) in a gradient mode (given in Table 2). The samples were processed with a 10 microlitre injection volume, a flow rate of 0.5 mL/min, run time of 11-minute duration, and a temperature

of 30°C during the study. The drug and associated contaminants were detected and their purity established using a PDA detector with a detection wavelength of 247 nm.

Table 2. The gradient program of validated method

No	Time (min)	A (%)	B (%)
1	0.01	50.0	50.0
2	3.0	50.0	50.0
3	5.0	80	20
4	8.0	50	50
5	10.0	50	50

A- KH_2PO_4 buffer (pH 2.5), **B** – Methanol

2.4. Preparation of Baloxavir Marboxil Standard Stock Solution

In a state of analytical balance 50 mg of Baloxavir marboxil standard was carefully weighed and transferred into a 10 mL dried volumetric flask, where it was dissolved with 7 mL of diluent (see section 2.8) and sonicated for 10 minutes. Then made up the final volume with diluent and filtered through a 0.45 micron filter to obtain a 5000 µg/mL solution. 0.2 mL of the aforesaid stock solution was transferred to a 10 mL volumetric flask and diluted with diluent to achieve a concentration of 100 ppm.

2.5. Preparation of Impurities Stock Solution

Five milligrams of each of the impurity working standards 1, 2, 3, 4, and 5 were accurately weighed and transferred to separate 10 mL clean and dried volumetric flasks, together with 7 mL of diluent (see section 2.8), which was sonicated for 10 minutes to dissolve. The diluent was used to make up the final volume. Then 1 mL of Imp - 1 solution was pipetted into a 10 mL volumetric flask, followed by 0.5 mL of Imp - 2, 3 mL of Imp - 3, 1.5 mL of Imp - 4, and 1 mL of Imp - 5 solutions in to the same flask, and then final volume was made with diluent.

2.6. Preparation of Standard Spiked Solution

One mL of the Baloxavir marboxil standard stock solution was transferred into 10 mL volumetric flask, then 3 mL of diluent (see section 2.8) and added 1 mL of impurities stock solution and further made up to the mark with diluent. The solution was filtered using with 0.45 µ syringe filter (Conc. of Baloxavir marboxil of 500 µg/mL, Conc. of Imp - 1 of 5 µg/mL, Imp - 2 of 2.5 µg/mL, Imp - 3 of 15 µg/mL, Imp - 4 of 7.5 µg/mL and Imp - 5 of 5 µg/mL).

2.7. Preparation of Mobile Phase

1000 mL of Methanol is taken as mobile phase A and 1000 mL of KH_2PO_4 buffer solution of pH 2.5 was taken as mobile phase B. These solutions were sonicated for 30 min. to degas. The mixture of solutions A and B in various proportions (gradient) was used as mobile phase.

2.8. Preparation of Diluent

KH_2PO_4 buffer (pH 2.5) and methanol were mixed in the composition of 50:50 and sonicated for 30 min. to gas and used as diluent.

2.9. Preparation of Blank Solution

Phosphate buffer (pH 2.5) and methanol were taken and mixed in the ratio 70:30. The solution was placed into HPLC as blank and recorded the chromatogram.

2.10. Method Validation

The established and optimized RP-HPLC method was validated in accordance with ICH recommendations Q2(R1) to evaluate the system appropriateness characteristics, precision, linearity, detection limit (LOD), quantification limit (LOQ), ruggedness, accuracy, and robustness.

2.10.1. System Suitability

To verify the system performance, system appropriateness study was performed by injecting standard solutions in six replicates in the chromatograph, and the chromatograms were documented. Parameters like theoretical plate count, peak symmetry and resolution between the peaks were determined.

2.10.2. Specificity

The specificity of the devised method was tested by injecting distinct solutions of blank, placebo, Baloxavir marboxil working standards, and related contaminants to inspect interferences from the obtained peaks.

2.10.3. Precision

Six replicates of samples of similar concentrations of Baloxavir marboxil and related impurities in the same laboratory were employed into chromatograph and calculated the percent assay and percent RSD for every component, thus the repeatability/ method precision was assessed. Repeatability/method precision was evaluated by injecting six replicates of similar concentrations of Baloxavir marboxil and related impurities in the same laboratory and calculated the % assay and RSD % for every compound.

2.10.4. Accuracy

Spiking method was employed for evaluating the recovery investigations. These studies were performed by adding known amounts at 50%, 100% and 150% levels of the working standard solutions of Baloxavir and related impurities to the pre-analysed sample which were injected into system in triplicates for accuracy study.

2.10.5. Linearity

The linearity of the standard Baloxavir marboxil and associated impurities solutions was determined by analysing various concentrations of the standard Baloxavir marboxil and related impurities solutions. Six working standard solutions for BXML and its five impurities, ranging from 50 to 750 µg/mL, were produced and injected. The acquired data were a linear function of concentration over area response, and the equation for calibration and correlation coefficient were calculated using linear least-squares regression analysis.

2.10.6. Limit of Detection (LOD) and Limit of Quantification (LOQ)

S/N ratio method was utilized for establishing the values of LOD and LOQ of Baloxavir marboxil and its related contaminants.

2.10.7. Robustness

The experimental settings were slightly changed to test the resilience of the proposed method. Baloxavir marboxil and its impurity peaks were investigated for characteristics such as separation factor, peak symmetry, and number of theoretical plates. The flow rate was changed by 0.1 mL/minute to test the outcome of the improved approach. At 5 °C, the effect of column temperature on the procedure was tested, and the mobile phase was changed by 5% from the organic phase's actual part. The aqueous fraction of the mobile phase was kept persistent in all of the above changed circumstances.

2.10.8. Forced Degradation Studies

Stress degradation tests were established by using working standard solutions of Baloxavir marboxil and its impurities with concentrations of 1 µg/mL each to give the optimised method's stability-indicating ability and specificity. Photolytic degradation (1.2 million lux hrs followed by 200 Watt hours), thermal degradation (maintained at 120 °C for 6 hrs), acidic stress (0.5 Normal hydrochloric acid for 30 minutes at 60 °C), alkali stress (0.5N sodium hydroxide for 30 min at 60 °C), oxidative stress (20 percent peroxide for 30 minutes at 60 °C), and neutral condition (refluxed for 12 hours at 60 °C) were all used to achieve intended degradation. The solutions were injected into the HPLC instrument, and the chromatograms obtained were examined to determine the sample's stability.

3. Results and Discussion

3.1. System Suitability

Imp -1, Imp -2, Baloxavir marboxil (BXML), Imp -3, Imp -4, and Imp -5 were shown to have retention durations of 1.919, 3.264, 4.429, 7.053, 8.247, and 8.937 min respectively. The column efficacy for BXML and impurity peaks was determined using a count of more than 3000 theoretical plates, with a tailing factor of less than 2.0 percent. The RSD of six replicate injections was determined to be under 2.0%. The chromatogram was presented in Figure 3 with the resolution of the peaks of BXML and impurities determined to be within the limits (>1.5).

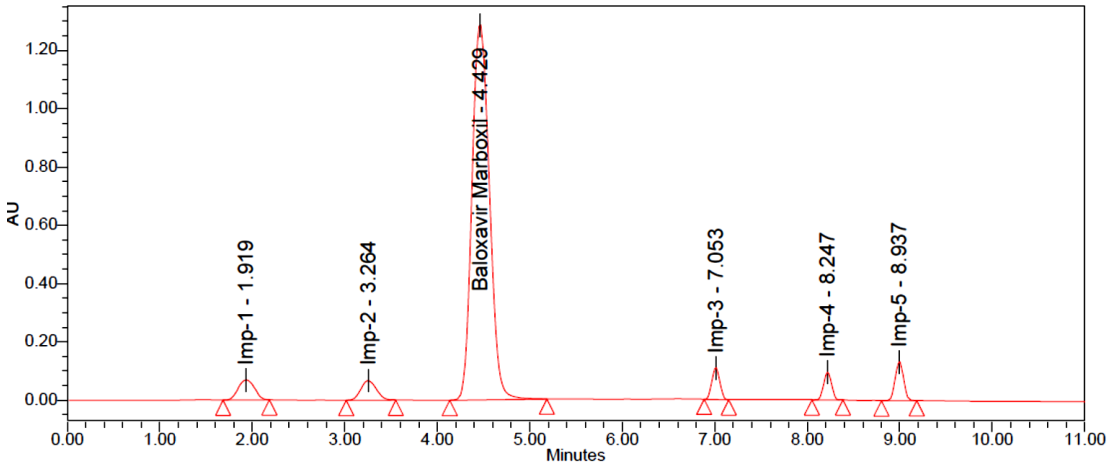

Figure 3. Chromatogram of mixture of Baloxavir marboxil with impurities

3.2. Specificity

The resultant chromatogram of placebo (Figure S2, see supporting information) shows that no peaks co eluted at the retention time of Baloxavir marboxil, as well as at the retention duration of known five impurities, indicating that the analyte peak was pure and that the other ingredients in the dosage form did not interfere with the analyte of interest.

3.3. Precision

From the obtained results represented in Table 3, it is verified that % assay for Baloxavir marboxil and impurities was found to be in the range of 98 – 102 %, and the % RSDs for Baloxavir marboxil and impurities are less than 2 %.

Table 3. The findings of the validation criteria are summarized below

Parameter	**Imp -1**	**Imp -2**	**Baloxavir marboxil**	**Imp -3**	**Imp -4**	**Imp -5**
Plate count	3597	6709	52622	27353	37520	41723
RSD %	0.303	0.371	1.074	0.115	0.527	0.433
Peak tailing	1.29	1.54	1.36	1.29	1.35	1.39
Resolution	-	4.38	3.61	9.47	7.45	4.69
Precision (% assay)	99.73 0.201	100.31	99.85	100.53	100.01	100.11
RSD %)		0.272	1.130	0.388	0.233	0.344
Accuracy (Mean % recovery)	99.9	99.9	99.7	99.6	99.5	100.2
Linearity (µg/mL)	0.50-7.50	0.25-3.75	50-750	1.50-22.50	0.75-11.25	0.50-7.50
Slope	160250.76	296100.73	33084.99	42766.28	73816.86	158923.19
Intercept	19992.03	2981.10	13785.63	11258.12	212.78	11352.66
Squared correlationcoefficient	0.99921	0.99935	0.99986	0.99977	0.99917	0.99930
LOD (µg/mL)	0.002	0.001	0.020	0.005	0.002	0.002
LOQ (µg/mL)	0.006	0.003	0.066	0.016	0.006	0.006

3.4. Accuracy

From the results summarized in Table 3, it is proved that the % recovery for Baloxavir marboxil and impurities found to be in the range of 98 –102 % and the % RSD for BXML and impurities is not more than two percent.

3.5. Linearity

Standard solutions at various concentrations were analyzed for linearity, and the results were presented in Table 3. The squared R^2 was more than 0.999, as inferred. The values of slope and y-intercept obtained from linearity plots revealed that the relationship between area response values and concentration is linear. Figure S3 (see supporting information) depicts the linearity plots of Baloxavir marboxil and its five associated impurities.

3.6. LOD and LOQ

By spiking all known contaminants and Baloxavir marboxil in the excipients solution, the limit of detection and limit of quantification of BXML and impurities (Imp - 1 to Imp -5) were obtained using the signal-to-noise (S/N) ratio method. Table 3 shows the values of the LOD and LOQ (Figure S4 and Figure S5, see supporting information).

3.7. Robustness

From the results it is evident that the system suitability characteristics such as separation, percentage RSD, peak asymmetry and the number of theoretical plates of BXML and impurities stayed unchanged by slight variation in the optimized conditions. As a result, the proposed approach was resistant to variations in the applied circumstances.

3.8. Results of Forced Degradation Studies

The samples were tested using the above-mentioned HPLC settings and a PDA detector to check for homogeneity and purity of the medication BXML as well as contaminants. The degradation was not seen in photolytic stress, hydrolysis, or thermal stress investigations, according to the results provided in Table 4, 5 and Figure S6-Figure S21 in supporting information. All of the peaks owing to acidic, alkali, oxidative, and reductive degradation were well separated from the peaks of Baloxavir marboxil and associated compounds, which was fascinating. Furthermore, based on evaluation factors such as purity angle and purity threshold, the peak purity of BXML and impurities was shown to be homogeneous. Peak purity verification demonstrates that no degradants are present, allowing for error-free quantification of Baloxavir marboxil and related compounds.

Table 4. Results of stress degradation study

Condition of Stress	Percent Degradation Baloxavir marboxil	Angle of purity	Threshold of purity
Control	0.02	1.774	4.918
Acid	12.64	1.718	4.946
Base	11.46	1.625	4.854
Peroxide	14.75	1.726	4.924
Reduction	13.29	1.751	4.962
Thermal	6.45	1.736	4.974
Photo Stability	2.24	1.732	4.915
Hydrolysis	2.68	1.748	4.964

Table 5. Results of stress degradation study of impurities

Condition of stress	Percent Degradation	Angle of purity	Threshold of purity
	Impurity 1		
Control	0.00	2.432	53.313
Acid	2.67	2.458	53.305
Base	2.22	2.246	53.325
Peroxide	3.67	2.463	53.311
Reduction	1.57	2.433	53.326
Thermal	1.26	2.442	53.348
Photo Stability	0.56	2.421	53.356
Hydrolysis	0.41	2.422	53.321
	Impurity 2		
Control	0.01	1.811	49.262
Acid	5.41	1.834	49.232
Base	5.36	1.326	49.465
Peroxide	2.87	1.874	49.236
Reduction	3.49	1.842	49.274
Thermal	2.07	1.874	49.253
Photo Stability	1.58	1.806	49.224
Hydrolysis	1.29	1.816	49.258
	Impurity 3		
Control	0.00	0.965	3.154
Acid	4.65	0.979	3.118
Base	4.21	0.125	3.425
Peroxide	3.88	0.932	3.163
Reduction	1.26	0.974	3.157
Thermal	1.57	0.928	3.169
Photo Stability	0.94	0.917	3.145
Hydrolysis	0.67	0.934	3.143

Table 5 continued..

Impurity 4			
Control	0.01	2.828	20.708
Acid	4.15	2.843	20.734
Base	3.88	2.569	20.663
Peroxide	5.21	2.854	20.768
Reduction	3.01	2.856	20.742
Thermal	1.11	2.853	20.783
Photo Stability	0.89	2.849	20.784
Hydrolysis	0.57	2.865	20.728
Impurity 5			
Control	0.01	8.232	90.511
Acid	4.26	8.216	90.574
Base	3.98	8.475	90.285
Peroxide	5.11	8.273	90.555
Reduction	2.67	8.241	90.569
Thermal	1.02	8.261	90.524
Photo Stability	0.54	8.263	90.535
Hydrolysis	0.37	8.227	90.549

4. Conclusion

For the simultaneous analysis of Baloxavir marboxil and its five known related compounds in a single run, a specific, simple, linear, exact, and durable RP-HPLC approach has been created. As per ICH requirements, the proposed technique was validated for system appropriateness, specificity, precision, stability, linearity, LoD, LoQ, accuracy, and robustness. Within proper limits, the suggested approach may measure known contaminants in the presence of unknown impurities. Thus, the results demonstrate the efficacy of the RP-HPLC method for separating five contaminants from Baloxavir marboxil, which can be used in the analysis of related compounds in Baloxavir marboxil Tablet formulations in pharmaceutical enterprises.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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