RESEARCH ARTICLE

AQbD Assisted Bioanalytical Method Development and Validation for Simultaneous Estimation of Montelukast and Bilastine by LC-MS Technique

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Received: 10th October, 2023; Revised: 12th November, 2023; Accepted: 27th November, 2023; Available Online: 25th December, 2023

ABSTRACT

This study describes how to use internal standards (IS) under the Analytical Quality by Design (AQbD) methodology to measure montelukast and bilastine in artificial rabbit plasma. The protein precipitation method was used for sample preparation and extraction. The chromatographic analysis was performed on the processed materials. The measurement range of the calibration was between 2 to 40 ng/mL for Bilastine and 1 to 20 ng/mL for montelukast. Optimizing mobile phase composition, flow rate, and pH were used to determine peak area and retention time. A significant reduction in method variability was observed as a result of the method of experimental design during optimization, and this enhanced the stability of the technique. A validation study demonstrated linearity, accuracy, precision, selectivity, sensitivity, and stability for estimating bilastine and montelukast in rabbit plasma using the developed method. The chemical composition of the drug was not altered during tests on its stability in human plasma. Overall, the investigations showed that the established approach is straightforward, reliable, and affordable for use in regular pharmacokinetic and bioequivalence research.

Keywords: Bilastine, Montelukast, AQbD, LC-MS, Rabbit plasma, Validation.

International Journal of Pharmaceutical Quality Assurance (2023); DOI: 10.25258/ijpqa.14.4.44

How to cite this article: Gnanasekaran D, Gandhimathi R. AQbD Assisted Bioanalytical Method Development and Validation for Simultaneous Estimation of Montelukast and Bilastine by LC-MS Technique. International Journal of Pharmaceutical Quality Assurance. 2023;14(4):1100-1106.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Asthma, exercise-induced bronchoconstriction, and seasonal allergic rhinitis can be treated with montelukast, a leukotriene receptor antagonist. Chronic spontaneous urticaria and seasonal allergic rhinitis can both be treated with bilastine, a peripheral histamine H1-antagonist. With the use of montelukast sodium in combination with bilastine, asthma-related allergic rhino conjunctivitis is treated.[1,2] The chemical structure of both montelukast and bilastine were described in Figure 1 (A and B).

The upcoming ICH Q-14 guideline emphasizes the need to understand and control the behavior of analytical methods in order to produce desired results based on predefined specifications and intended uses. Analytical techniques must be used according to predefined specifications and the intended use to produce the desired results. An enhanced analytical quality-by-design methodology that assesses risk can be employed to determine how samples behave according to ICH Q-8(R2), Q-9, and Q-10.[3-6]

The quantification of montelukast and bilastine has been reported using a variety of analytical techniques, including UV,[7,8] RP-HPLC,[9-12] and HPLC.[13,14] It should be noted that no QbD-based optimization strategy using rabbit plasma samples by Liquid chromatography-mass spectrometry (LC-MS) method has been created and published in the literature.

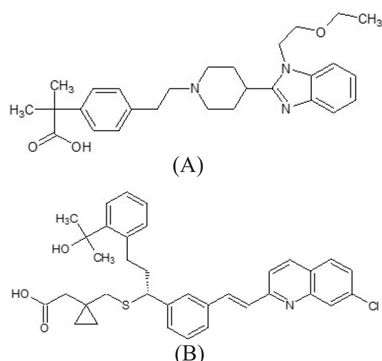

Figure 1: Structure for Analytes 1 (A) montelukast 1(B) bilastine

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In order to estimate drugs in biological samples using LC-MS, expensive solvents must be used, column temperatures must be maintained and other chromatographic conditions must be maintained, as well as laborious extraction procedures must be used. To develop a method, significant resources, costs, and efforts must be dedicated.[15-17]

The analytical QbD paradigm has increasingly been used for *in-vitro* and *in-vivo* drug estimation.[18-20] A wide range of domains, including drug design, formulation development, and analytical development practice, can benefit from such an approach.[21-23] Application of QbD technique can greatly benefit both the understanding of LC-MS method development variability and the development of analytical and bioanalytical chromatographic methods.

A systematic chromatographic method is developed by designing experiments (DoE) based on QbD. By using this method, method variables that play a significant role in method performance can be identified and optimized to reduce the time, effort, and cost associated with method development. The QbD methodology has demonstrated greater success in the creation of chromatography methods with greater flexibility and improved performance in the literature.[24,25]

As the first systematic method creation and optimization study using QbD methodology, this research work represents a unique contribution to the field. We explored new solvent systems by measuring bilastine and montelukast with LC-MS.[26] A chosen experimental layout was applied to refine and validate the created method for routine application in biological sample analysis in accordance with ICH guidelines.

MATERIALS AND METHODS

Pure API of bilastine and montelukast were obtained samples from Biocon, Bangalore. HPLC grade of methanol, acetonitrile and water (milli Q or Equivalent) were purchased from M C I Pvt. Limited, India.

LC-MS Conditions

LC solution software, Pump LC2010 binary and PDA detectors were used with Shimadzu HPLC2010 CHT separation module. A Kinetex column with an id of 100 mm and a particle size of 2.6 mm was used to separate the compounds. 80% v/v methanol and buffer (pH 2.5) were used for the mobile phase. It took 5 minutes to run the experiment at 0.5 mL/min. An injector loop from Rheodyne was used for injection and a 254 nm detector was used for detection. Using an HPLC system and a turbo-ion spray positive ion source, the Sciex API 4000 mass spectrometer is used as the mass spectrometer. It was used to detect multiple reactions using multiple reaction monitoring (MRM). At 5500V, the spray get is maintained at approximately 550°C. To obtain high-purity nitrogen gas, MG Industries obtained ultra and zero-grade nitrogen gas. In addition to curtain gas, it was used as auxiliary gas flowing into the turbo at 0.8 mL/min.

Preparation of Standard Solution

It was prepared in mobile phase stock standards of bilastine (20 ng/mL) and montelukast (10 ng/mL). Stored between 2 and 8°C, the prepared solution was used as soon as possible. In preparation for the analysis, we diluted the stock standard solutions with mobile phase to make the working standard solutions.

Sample Solution Preparation

Weighing and powering twenty tablets was completed. It was calculated that 10 mg of tablet powder should be used in a volumetric flask containing 10 mL per mg (1-mg/mL). A 10-minute sonication was performed on this mixture. The solutions were labelled and stored at 0–4°C when not in use and expiry was 2 weeks after the date of preparation.

Prepare Standards For The Calibration Curve (Cc) As Well As The Quality Control Standard (Qc).

Two analytes were prepared in CH_3OH, and stored at 4°C in the presence of one milligram/mL of methanol per analyte. Stock solution aliquots were spiked into drug-free plasma at a final concentration of 200 to 500 g/mL to determine the plasma calibration standards. A quality control sample was prepared by aliquoting drug solutions and adding them to drug-free plasma.[27] The QC samples were LLQC, LQC, MQC and HQC aliquot (1–20 µg/mL). Plasma sample (200–500 µg/mL) was aliquot into a centrifuged tube and spiked. The samples were centrifuged for 15–20 minutes at 4000 rpm.

Plasma Sample Preparation

Bilastine and Montelukast solutions were used to achieve 500 g/mL analyte concentrations in spiked blank plasma. In the following steps, 3 mL of C6H5CH3 extraction solvent and 100 L of potassium hydroxide were added, followed by alkalization with 100 µL of 3M KOH. Centrifuge the mixture at 3500 rpm for 5 minutes after gentle shaking for 5 minutes. Under vacuum at 60°C, the supernatant organic layer was evaporated to dryness using an Eppendorf concentrator. Injecting 20 µL of the solvent into the chromatography system after reconstitution with 100 µL of mobile phase and 30 seconds of vortexing. By comparing analytes spiked with different analytes and the blank plasma sample before evaporation with analytes spiked with different analytes, the recovery percentage of the drug was estimated.

Optimization Procedure (Experimental Design and Analysis)

Analyze Bilastine and Montelukast in rabbit plasma via LC-MS/MS. LC-MS/MS separations can be optimized with CCD because of its flexibility and ability to analyze the factors involved and their interactions. Using prior knowledge and preliminary research, the key factors were identified. In order to optimize the reaction, three factors were selected: the MeOH concentration, the buffer pH, and the flow rate. As response factor response 1 for first peak i.e., Bilastine (Rt), response 2 the retention time of second peak Montelukast (Rs), and response 3 plate count were chosen.

Method Validation

Validation was performed in plasma by analyzing the typical chromatograms for bilastine Montelukast, as defined in the Bioanalytical method validation. It took 1.85 minutes for Bilastine to bind to the cell and 2.29 minutes for Montelukast

Table 1: Arrangements and responses related to central composite design

Std	*Run*	*Type*	*Factor-1 Organic solvent (%) (A)*	*Factor-2 (Buffer: pH(B))*	*Factor-3 (Flow rate (C))*	*Response-1 Rt mins*	*Response-2 Rs mins*	*Response-3 Plate count*
1	1	Center	20	2.5	0.5	1.887	4.08	8745
2	2	Center	20	2.5	0.5	1.887	4.08	8745
3	3	Center	20	2.5	0.5	1.887	4.08	8745
4	4	Center	20	2.5	0.5	1.887	4.08	8745
5	5	Center	20	2.5	0.5	1.887	4.08	8745
6	6	Center	20	2.5	0.5	1.887	4.08	8745
7	7	Axial	20	3.34	0.331821	2.021	3.89	8626
8	8	Axial	20	2.5	0.5	2.054	3.95	8556
9	9	Axial	28.409	1.65	0.5	1.541	4.12	8896
10	10	Axial	20	2.5	0.5	1.724	4.02	8745
11	11	Axial	20	2.5	0.668179	1.681	4.11	8898
12	12	Axial	11.591	3	0.5	2.526	4.27	8823
13	13	Factorial	25	2	0.4	1.975	4.12	8521
14	14	Factorial	15	3	0.6	2.387	4.56	8896
15	15	Factorial	15	2	0.4	2.613	3.18	8745
16	16	Factorial	25	3	0.4	1.962	3.54	8623
17	17	Factorial	15	2	0.6	2.458	3.57	8771
18	18	Factorial	15	2	0.4	2.587	3.27	8989
19	19	Factorial	25	3	0.6	1.691	4.28	8966
20	20	Factorial	25	2	0.6	1.841	4.02	8497

to bind to the cell. A chromatogram of the sample did not reveal any interfering endogenous peaks. Prior to the analytical run, a set of reference standards was analyzed to determine whether the system is suitable by determining its performance.

Luminescence chromatograms were compared between six blank matrices and a blank spike to determine the specificity.

Based on calibration standards, replicate analyses (n = 6) were used to study the sensitivity of the developed method for bilastine and montelukast LLQC concentrations of 0.501 and 0.250 ng/mL, respectively. Applying a weighting factor of 1/C, three comprehensive standard curves were constructed, covering Bilastine concentrations from 2 to 40 ng/mL and Montelukast concentrations from 1 to 20 ng/mL, to assess linearity. Precision and accuracy were evaluated by analyzing duplicate sets of QC samples at four concentrations (LLoQ, LQC, MQC, HQC). To assess the matrix effect on plasma constituents post-ionization of analytes (n = 6), a matrix analysis of plasma constituents was conducted. Samples, containing 20 ng/mL bilastine and 10 ng/mL montelukast, were analyzed directly. The matrix effect in the intended method was evaluated using chromatographically screened rabbit plasma. As a standard procedure, all stability determinations should be conducted using samples prepared from QC solutions fresh from the laboratory.

RESULTS AND DISCUSSION

We used a central composite design forliquid–liquid extraction to analyze analytes in plasma by LC–MS. LC-MS separations were optimized by gaining a deeper understanding factors involved and their interactions by applying CCD. A selection of factors and responses has been made. As can be seen in Table 1, the factors and response variables have different ranges.

When there are three factors in the experimental design, which include cross terms, linear and quadratic the model should look like this:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

Where,

Y –Modeled response,

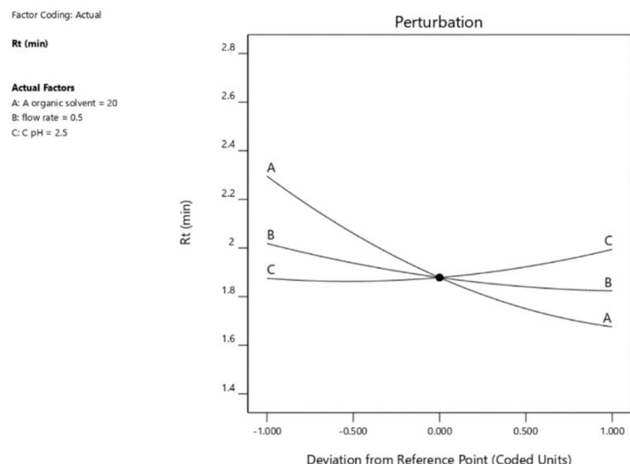
β- Regression Co-efficient

X_1, X_2 and X_3 = factors A, B and C respectively.

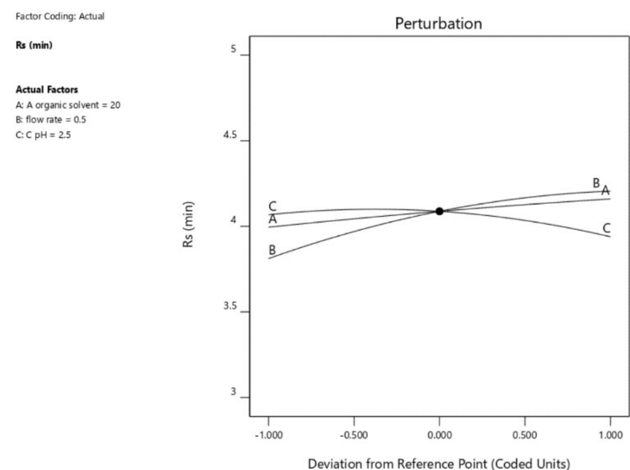
In order to create an easily understandable and realistic model, backward elimination was used to eliminate unimportant terms. When regressor variables are removed from statistical models, R2 declines due to a decline in adjusted R2, which takes these variables into account.[26] In the experiment, the adjusted R2 values of 0.81 were found to be satisfactory for fitting the experimental data to the second-order polynomial equations.[27] Among all the reduced models, p-values greater than 0.05 were significant. A precision value is used to calculate the signal-to-noise (response) ratio. It is preferable to have a ratio of at less than four.[28,29] According to the model; there was sufficient signal for separation at a ratio between 6.57 and 10.29, which indicated a significant separation. Models that have coefficients of variation (CV) less than 10% are considered reasonably reproducible. Reproducibility is measured by this metric. Table 2 shows that BC (+0.2175) of the Rs model had

Table 2: Reduction of the response surface in ANOVA models reveals the following statistical parameters

Response	*Regression model*	*Adjusted R2*	*p-value*	*(%) C.V*	*Adequate precision*
Rt	+1.88-0.3098*A -0.0974* B + 0.0596*C 0.0064*AB + 0.0084*AC + 0.0229*BC -0.1072* A^2 + 0.0426* B^2 +0.0561*C^2	0.8028	< 0.0001	7.64	10.2912
Rs	+4.09+0.0826* A +0.1970*B - 0.0643* C -0.1300* AB +0.1750* AC -0.2175* BC -0.0103* A^2 -0.0792* B^2 -0.0845* C^2	0.8317	< 0.0001	5.79	6.9702
Plate count	+8745.23-49.15*A+51.95*B-92.10*C+48.25*AB-25.25*AC-31.00*BC+38.99*A^2+4.52*B^2-34.91*C^2	0.8126	< 0.0001	12.17	6.5715



(a): Retention time



(b): resolution factor

Figure 2: Perturbation plot

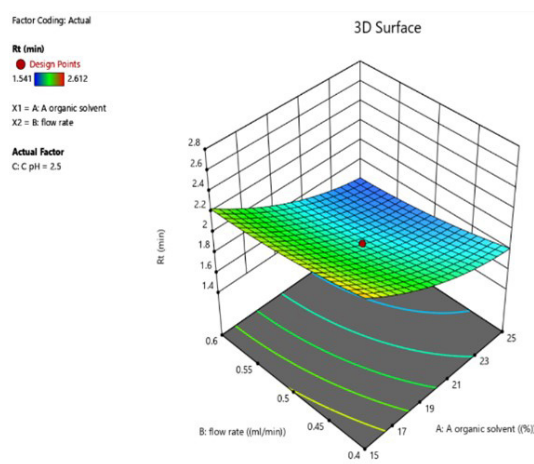


Figure 3 (a): Retention time Rt

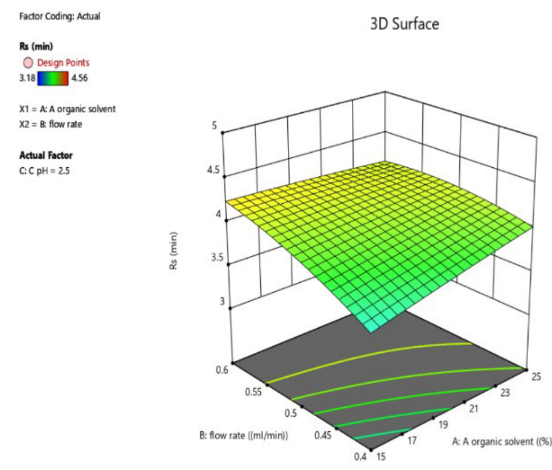


Figure 3 (b): Resolution time Rs

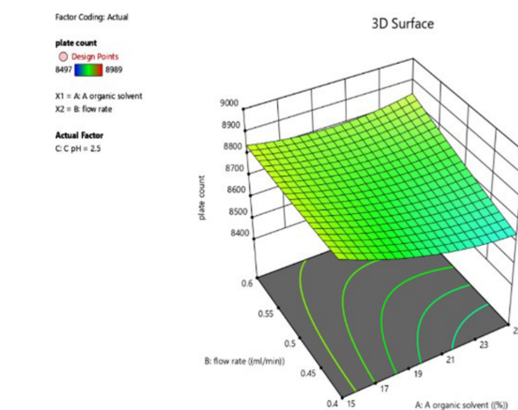


Figure 3(c): Plate count

the highest absolute coefficient among the models fitted. A statistically significant interaction exists between B and C for Rs ($p < 0.0001$). To optimize chromatographic separation, multifactor research is crucial. Figures 2 (a and b) and 3 (a and b) show three-dimensional response surfaces and perturbation plots that can help the reader better understand the results. Plots of response surfaces were generated from fit models with the largest delta coefficients based on the quadratic and interaction terms. An Rt and Rs response plot is most commonly constructed with factors A and C at pH 0.5, with factor B held constant. We were able to understand how buffer pH and flow rate affected analysis time with the three-dimensional charts.

Table 3: Optimizing the individual responses based on a set of criteria

Response	*Lower limit*	*Upper limit*	*Criteria/Goal*
Rt	1.541	2.612	minimize
Rs	3.18	4.56	minimize
Plate count	8497	8989	Is in range

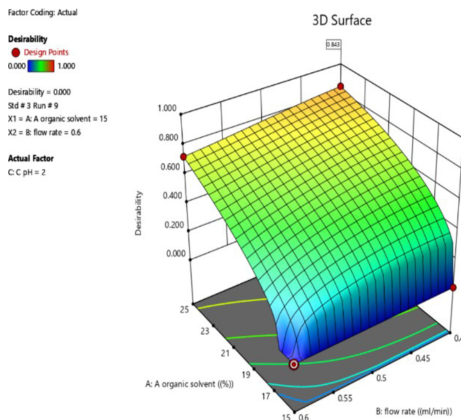


Figure 4: An overview of the desirability function is represented graphically

In this plot, perturbation plots are plotted above the response surface plot to illustrate how the response changes as the elements vary around the reference point.

Depending on the slope or curve of the curve, the response is more sensitive. The most significant impact was felt by buffer pH (Factor-B), followed by buffer pH (Factor-C), and finally by buffer pH (Factor-A). In addition to methanol concentration and flow rate, Rt and Rs were significantly affected by these two variables. In the presence of increased Rt and Rs values, methanol concentrations (factor A) increase, whereas in the presence of decreased Rt and Rs values, methanol concentrations (factor C) decrease. Factor A, methanol concentration, played a relatively minor role in the separation effect, according to disturbance plots and response surfaces. An optimization

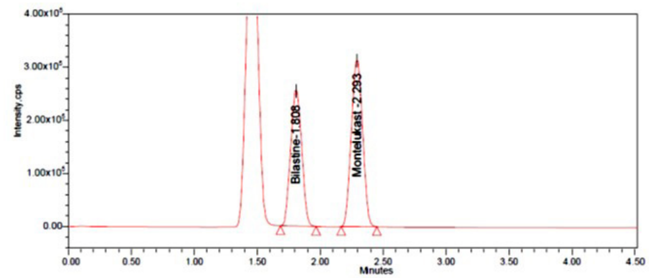


Figure 5: Optimized chromatogram

process was followed for each response, as shown in Table 3.

The above table showed that under the column criteria, Rt was minimized to shorten the analysis time while Rs was minimized so that bilastine and montelukast could be separated based on baseline performance. Depending on the importance level, target values could be prioritized from 1 to 5. An optimization procedure was conducted based on the above conditions and restrictions. Figure 4 shows the response surface of the global desirability function.

According to the optimal coordinates (D = 0.843), the methanol concentration should be 21.87%, the buffer pH should be 2.0, and the flow rate should be 0.40 mL/min. The mobile phase consisted of methanol and buffer pH 2.3 (21.87:77.13 v/v) as the detection solvent, and a flow rate of 0.40 mL/min was utilized with Kinetex columns (100 x 4.6 mm, 2.6 mm particle size). Mass spectroscopy was used to use positive ion sprays and multireaction monitoring (MRM). Table 4 summarizes experimental and predicted responses under these conditions based on the optimized chromatogram shown in Figure 5.

METHOD VALIDATION

The suitability of the system performance was tested, and the results were found to be satisfactory. Table 5 shows the reports. The retention times or ISTD, were not observed to interfere with any of the six random blank rabbit plasma samples. The reports of analysis were shown in Tables 6 and 7

Table 4: A comparison of experimental and predicted values for various functions has been conducted under optimal conditions

Optimum conditions	*Methanol (%v/v)*	*Buffer (pH)*	*Flow rate (ml/min)*	*Rt*	*Rs*	*Plate count*
Predictive	21.87	2.3	0.4	count	3.60	8690.83
Experimental	21.87	2.3	0.4	1.80	3.54	8816.68
Average error				1.63	1.66	1.44
Desirability value= 0.843						

Table 5: System suitability

System suitability	*Bilastine MQC(20ng/mL)*	*Montelukast MQC(10ng/mL)*
Mean Analyte Area(cps)	3.05 x 10^5	1.54 x 10^5
Mean Analyte RT(min)	1.806	2.296
Mean ISTD Area (50ng/mL)forBilastine and (10ng/mL) for Montelukast	3.04 x 10^5	1.53 x 10^5
Mean ISTD RT (min)	1.809	2.296
Mean Area Ratio	0.998	1.005

Table 6: An analysis of the biological matrix of bilastine and its specificity

Sample ID LLoQ (0.5 ng/mL)	*Intensity(cps)*		*% Interference*		*Pass/ Fail*
	Drug	*ISTD*	*Drug*	*ISTD*	
LLoQ 1	0.511×10^5	3.018×10^5	0	0	Pass
LLoQ 2	0.513×10^5	3.015×10^5	0	0	Pass
LLoQ 3	0.511×10^5	3.014×10^5	0	0	Pass
LLoQ 4	0.514×10^5	3.011×10^5	0	0	Pass
LLoQ 5	0.515×10^5	3.016×10^5	0	0	Pass
LLoQ 6	0.517×10^5	3.019×10^5	0	0	Pass

Table 7: Specificity and screening of biological matrix of montelukast

Sample ID LLoQ 1 (0.25ng/mL)	*Response*		*% Interference*		*Pass/ Fail*
	Drug	*ISTD*	*Drug*	*ISTD*	
LLoQ 1	0.0375×10^5	1.551×10^5	0	0	Pass
LLoQ 2	0.0378×10^5	1.554×10^5	0	0	Pass
LLoQ 3	0.0371×10^5	1.551×10^5	0	0	Pass
LLoQ 4	0.0372×10^5	1.556×10^5	0	0	Pass
LLoQ 5	0.0377×10^5	1.558×10^5	0	0	Pass
LLoQ 6	0.0374×10^5	1.557×10^5	0	0	Pass

Table 8: Method validation parameters results

Parameters	*Bilastine*	*Montelukast*
Range (ng/mL)	2–40	1–20
Y = mx+c	Y = 0.0497x + 0.00018	Y = 0.0996x + 0.00017
r^2	0.9998	0.9998
Slope	0.0497	0.0996
Intercept	0.00018	0.00017
Limit of detection (µg/mL)	3	10
Limit of quantification (µg/mL)	3	10
Precision and accuracy (Mean %CV for QC samples)	0.84	0.70
Sensitivity LLoQ (0.5010 ng/mL for bilastine, 250 ng/mL for montelukast) %CV	0.427	0.58
Matrix effect (18 runs, %Mean Accuracy, HQC and LQC Samples)	99.32	99.61

Standard curves were linear for both bilastine and montelukast at concentrations between 2 and 40 ng/mL. Figures 6 and 7 show the calibration curves. Both bilastine and montelukast yielded correlation coefficients of 0.999. A rabbit peak area comparison was used to quantify samples based on their analyte peak areas. The ratio of peak area to plasma concentration was plotted. Neither bilastine nor montelukast had a LoD value greater than 3. Bilastine and Montelukast both had LoQ values of 10. Bilastine and montelukast were analyzed at four different QC levels in six replicates to evaluate precision and accuracy. A chromatographically screened rabbit plasma was used to test the matrix effect intended method. Sensitivity results were found to be within the limit. The reports are shown in Table 8.

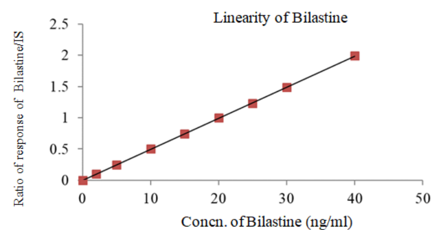


Figure 6: Calibration plot for Bilastine

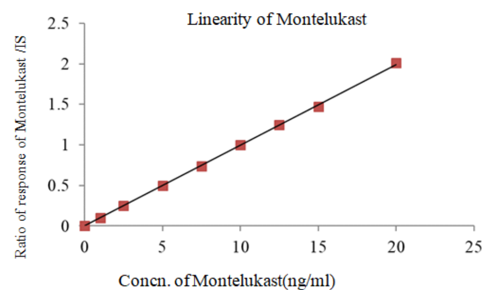


Figure 7: Calibration plot for Montelukast

Table 9: Report for Stability Studies

Stability Studies	*Bilastine*	*Montelukast*
Benchtop stability (6 runs, %Mean Accuracy, HQC and LQC Samples)	98.48	98.06
Auto sampler stability (24 runs, %Mean Accuracy, HQC, MQC and LQC Samples)	98.38	98.23
Freeze thaw stability (6 runs, %Mean Accuracy, HQC and LQC Samples)	98.06	98.0
Wet extract stability (6 runs, %Mean Accuracy, HQC and LQC Samples)	98.47	98.83

All stability studies reports %CV and mean accuracy were found within the limit. The reports are shown in Table 9.

CONCLUSION

Montelukast and bilastine were simultaneously measured in artificial rabbit plasma using the above developed method with good sensitivity and specificity. Endogenous compounds did not cause significant interferences or matrix effects. Optimal conditions for the factors were determined through the utilization of a central composite design approach in conjunction with response surface methodology. Utilizing Derringer's desirability function, a multifaceted decision-making tool, we concurrently optimized objectives, capacities, and analysis times. Response surface technique and experimental design produce a greater insight into how chromatographic factors affect separation assignments and their interconnections. Additionally, it gives the chromatograph the opportunity to customize the response to the analysis matrices based on their nature. Study results showed that selecting optimum conditions during the development of assay methods can be accomplished with the help of this approach. Hence, the experimental design approach proves to be a suitable tool for optimizing and developing LC-MS

methods with regard to time, cost, and laboratory resources. Routine clinical laboratory analysis can be carried out using an analytical method that has these characteristics.

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