IJPPR
INTERNATIONAL JOURNAL OF PHARMACY & PHARMACEUTICAL RESEARCH
An official Publication of Human Journals
ISSN 2349-7203
HUMAN

Human Journals
Research Article
June 2024 Vol.:30, Issue:6

Development and Validation of RP-HPLC Method for Simultaneous Estimation of Bilastine and Montelukast in Bulk and Pharmaceutical Dosage Form

IJPPR
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ISSN 2349-7203
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Submitted: 23 May 2024
Accepted: 28 May 2024
Published: 30 June 2024

Keywords: wavelength, Bilastine, Montelukast, HPLC.

ABSTRACT

An effort has been made to construct a Reverse Phase High Performance Liquid Chromatographic method for the estimation of Bilastine and Montelukast in bulk and pharmaceutical dosage form, as well as to validate the method that has been developed in accordance with the guidelines established by ICH Q2 (R1). Bilastine and Montelukast in bulk and pharmaceutical dose form were both subjected to the development of the RP-HPLC method for their respective estimations. Phenomenex Kinetex XB-C8 (150 mm × 4.6 mm, 5 mm) was utilized as the stationary phase for the quantification process. The mobile phase consisted of 0.1% perchloric acid and Acetonitrile [Gradient 1]. It was ensured that the flow rate of the mobile phase was kept at 270 nm and 1 ml per minute. Bilastine and Montelukast, two different medications, started to elute at 4.59 and 7.23 minutes, respectively.

INTRODUCTION:-

The theory of chromatography has been used as the foundation for System Suitability tests. These tests represent a set of quantitative criteria that evaluate the suitability of the chromatographic system to identify and quantify drug-related samples using high-performance liquid chromatography (HPLC) at any stage of the pharmaceutical analysis process.

When it comes to drug analysis, the analytical technique that is experiencing the most rapid expansion is high-performance liquid chromatography, which is also referred to as HPLC. The ease of use, high specificity, and broad range of sensitivity of this instrument make it a great choice for the analysis of a wide variety of pharmaceuticals in dosage forms as well as biological fluids. Because of these characteristics, the instrument is an outstanding choice. The method of liquid chromatography known as "high performance liquid chromatography" (HPLC) is a type of liquid chromatography in which the liquid mobile phase is pushed along the column at a rapid pace. Since this is the case, the amount of time required for analysis is cut down by one to two orders of magnitude in comparison to the conventional column chromatography method. Additionally, the utilization of much smaller particles of the adsorbent or support becomes conceivable, which has the potential to considerably boost the column efficiency [45]. This is a significant advancement in the field. There is a rapid increase in the significance of chromatography within the realm of pharmaceutical analysis. This is due to the fact that chromatography enables the accurate differentiation, selective identification, and quantitative determination of compounds that are composed of molecules that are structurally identical to one another.

HPLC Method Development

1. Chromatographic Conditions:

a. Oven Temp: 30°C

b. Flow rate: 0.5 ml/min.

c. Mobile Phase: 0.1% Perchloric acid : Acetonitrile

i. Preparation of 0.1% Perchloric acid: Take 1ml of Perchloric Acid in 1000 ml type I water.

d. Gradient Program:

Time (minutes)	0.1% Perchloric Acid (%)	Acetonitrile (%)
0.00	70	30
1.00	70	30
5.00	30	70
6.00	30	70
6.01	70	30
10.00	70	30

e. Runtime: 10 minutes

f. Injection Volume: 10µl

g. Wavelength: 270 nm

h. Diluent: 0.1% Perchloric acid: Acetonitrile (50: 50%, v/v)

i. Column : Phenomenex Kinetex XB-C8 (150 x 4.6 mm, 5 µm)

1. Standard Preparation:

a. Bilastine Standard Stock Solution-I (BSSS-I):

i. Initially Prepare a Standard Stock Solution (SSS-I) by adding10 mg of Bilastine in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent. (Conc. Of Bilastine = 1000 µg/ml).

b. Montelukast Standard Stock Solution-I(MSSS-I):

i. Then prepare a Standard Stock Solution (SSS-II) of Montelukast by adding 10mg in 10 ml volumetric flask & add 5 ml diluent, mix for 2 minutes and make the volume to 10 ml with diluent.(Conc. of Montelukast = 1000µg/ml).

c. Then add 1.0 ml of CSSS-I & 0.5 ml TSSS-I in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Bilastine=100µg/ml & Montelukast = 50µg/ml).

Validation of RP-HPLC Method:

d. **Specificity:**

i. Individual samples of Bilastine and Montelukast were prepared of 100µg/ml and 50µg/ml, respectively and peaks were for identified from Retention Time.

ii. Blank was injected to ensure there is no blank peak interfering with the main analyte peaks.

e. **Instrument Precision& System Suitability:**

i. A single sample was prepared as described and 6 injections were made from same sample and checked for system suitability.

ii. System suitability parameters are as below:

1. Retention Time,

2. Theoretical plates,

3. Asymmetry (Tailing factor),

4. Resolution.

f. **Linearity& Range:**

i. Samples of varying concentrations ranging from 80%-120% were made.

ii. The sample preparations and concentrations are given as below;

iii. X ml of Bilastine and Y ml of Montelukast were added to 10 ml diluent to make up the concentrations given above:

% Level	X ml of BSSS-I	Bilastine Conc. (µg/ml)	Y ml of MSSS-I	Montelukast Conc. (µg/ml)	Diluted to
80	0.8	80	0.4	40	10 ml
90	0.9	90	0.45	45	10 ml
100	1.0	100	0.5	50	10 ml
110	1.1	110	0.55	55	10 ml
120	1.2	120	0.6	60	10 ml

g. **Accuracy:**

i. Samples were prepared of 80%, 100% and 120% concentration by spiking the same amount of concentration given above in table for both Bilastine and Montelukast.

ii. Samples were injected in triplicate to calculate % RSD.

iii. % recovery was also calculated.

h. **LOD/ LOQ:**

i. Was calculated for both drugs by using ANOVA technique.

ii. Formula:

$$LOD = \frac{3.3 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

$$LOQ = \frac{10 \times Std.Error\ of\ Intercept}{Coefficients\ of\ X\ Variable\ 1}$$

i. **Robustness:**

i. The modification of the column temperature was used to make the robustness measure by ± 2˚C and Wavelength by ± 2 nm.

ii. Each Sample was injected in triplicate and % RSD of area at each condition was calculated. System suitability was also checked.

Condition	**Increased**	**Normal**	**Decreased**
Column Oven Temperature	32˚C	30˚C	28˚C
Wavelength	272 nm	270 nm	268 nm

j. **Inter & Intraday Precision:**

i. Single mixture working standard and drug product samples were prepared and injected twice in a day at different time intervals to evaluate intra-day precision.

ii. Same mixture working standard and drug product samples were analysed on second day to evaluate the inter-day precision.

iii. % Assay was calculated at each interval and stability of solutions were estimated.

7. Results and Discussion

7.1 Preliminary Analysis of Drug

Table 1: Preliminary Data of Drugs

Sr. No.	Properties	Bilastine	Montelukast
1.	**Description**	White to Creamy white coloured Powder	White to off white powder
2.	**Solubility**	Bilastine is slightly soluble in Methanol and DMSO and practically insoluble in water	Montelukast is freely soluble in water and very soluble in methanol and ethanol.
3.	**Melting Point**	The melting of Bilastine was found to be approximately 200℃.	The melting of Montelukast was found to be 147℃

HPLC Method for Bilastine and Montelukast

7.3.1. HPLC Method Development

Few important selections were done before initiating the development work. Stationary Phase was selected based on the polarity. Based on literature review, the mobile phase of 0.1% Perchloric acid and Acetonitrile was chosen for the HPLC analysis of Bilastine and Montelukast with Phenomenex Kinetex XB-C8 column with dimension 150 x 4.6 mm, 5 micron particle size. Column temperature at 30℃ and injection volume at 10 µl. Diluent as 50:50 0.1% Perchloric acid and Acetonitrile. The trials for method development are given are below:

Table 2: Method Development for Bilastine and Montelukast HPLC

Trial No.	Mobile Phase	Ratio	Diluent	Column	Flow rate	Wavelength
1	0.1% Perchloric acid:ACN	50-50	50 Water: 50 CAN	Phenomenex Kinetex XB-C8 (150 x 4.6mm, 5µm)	1.0 ml/min	250 nm
2	0.1% Perchloric acid:ACN	50-50	50 0.1% Perchloric acid: 50 CAN	Phenomenex Kinetex XB-C8 (150 x 4.6mm, 5µm)	1.0 ml/min	270 nm
3	0.1% Perchloric acid:ACN	60-40	50 0.1% Perchloric acid: 50 CAN	Phenomenex Kinetex XB-C8 (150 x 4.6mm, 5µm)	1.0 ml/min	270 nm
4	0.1% Perchloric acid:ACN	30-70	50 0.1% Perchloric acid: 50 CAN	Phenomenex Kinetex XB-C8 (150 x 4.6mm, 5µm)	1.0 ml/min	270 nm
5	0.1% Perchloric acid:ACN	Gradient 1	50 0.1% Perchloric acid: 50 CAN	Phenomenex Kinetex XB-C8 (150 x 4.6mm, 5µm)	1.0 ml/min	270 nm

Gradient 1 is given below:

	Time (min)	0.1% PA (%)	ACN (%)
Gradient 1	0.00	70	30
	1.00	70	30
	5.00	30	70
	6.00	30	70
	6.01	70	30
	10.00	70	30
	15.00	70	30

HPLC Results of Method Development:

Table 3: Method development results of Bilastine and Montelukast

Trial No.	Bilastine				Montelukast			
	RT	TP	Asymmetry	Resolution	RT	TP	Asymmetry	Resolution
1.	Not Detected				12.22	13252	1.37	0.00
2.	1.89	4839	1.30	0.00	7.92	11566	1.62	29.94
3.	1.53	4277	1.22	0.00	3.09	9355	1.38	14.07
4.	1.42	2099	1.00	0.00	2.10	6936	1.33	6.05
5.	4.57	20272	1.35	0.00	7.21	77444	1.23	22.77

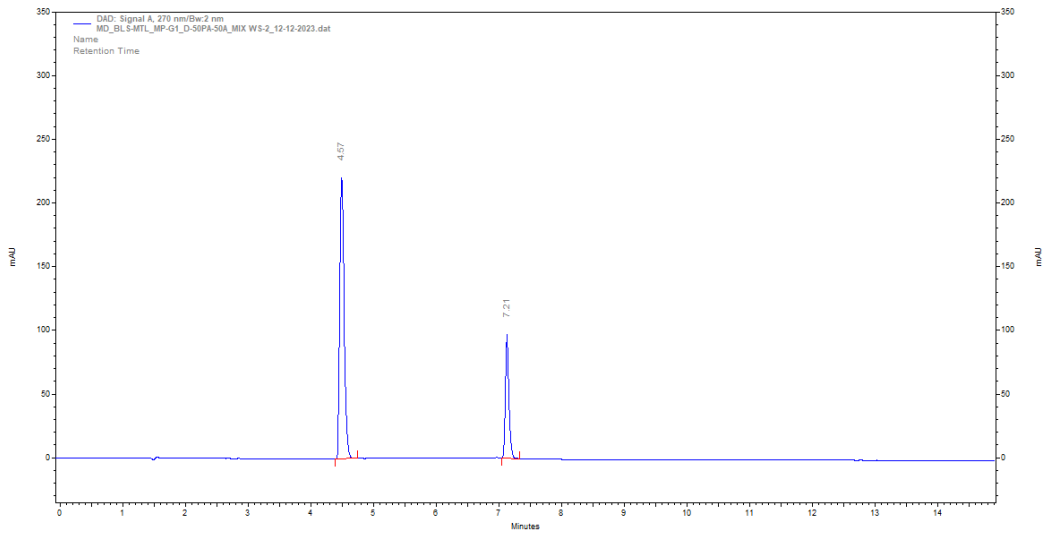


Figure 1: Method Development- Trial 5

Final Method of Analysis:

Trial 5 was selected as the final method.

Table 4: Final Chromatographic conditions

<table>
<tr><th>Parameter</th><th>Condition</th></tr>
<tr><td>HPLC Instrument</td><td>Agilent 1260 Infinity II</td></tr>
<tr><td>Column</td><td>Phenomenex Kinetex XB-C8 (150 mm x 4.6 mm, 5µm)</td></tr>
<tr><td>Wavelength</td><td>270 nm</td></tr>
<tr><td>Mobile Phase</td><td>Mobile Phase A –0.1% Perchloric acid
Mobile Phase B – Acetonitrile
(Gradient 1)</td></tr>
<tr><td>Gradient 1</td><td>
<table>
<tr><th>Time (min)</th><th>0.1% PA (%)</th><th>ACN (%)</th></tr>
<tr><td>0.00</td><td>70</td><td>30</td></tr>
<tr><td>1.00</td><td>70</td><td>30</td></tr>
<tr><td>5.00</td><td>30</td><td>70</td></tr>
<tr><td>6.00</td><td>30</td><td>70</td></tr>
<tr><td>6.01</td><td>70</td><td>30</td></tr>
<tr><td>10.00</td><td>70</td><td>30</td></tr>
<tr><td>15.00</td><td>70</td><td>30</td></tr>
</table>
</td></tr>
<tr><td>Diluent</td><td>0.1% Perchloric acid : Acetonitrile (50:50) v/v</td></tr>
<tr><td>Run time</td><td>15 minutes</td></tr>
<tr><td>Injection Volume</td><td>10 micro liters</td></tr>
<tr><td>Flow Rate</td><td>1 ml/min</td></tr>
<tr><td>Column oven Temperature</td><td>30°C (± 2°C allowed by Robustness)</td></tr>
</table>

The developed method can be used for individual estimation of Bilastine and Montelukast within 15 minutes.

Method Validation

a. Specificity

In order to determine whether or not there was any interaction between the peaks that came from the blank or the APIs, specificity was carried out.

Table 5: Specificity results of Bilastine and Montelukast

Sample	Bilastine			Montelukast		
	RT	Area	% Assay	RT	Area	% Assay
Bilastine	4.59	2221543	-	-	-	-
Montelukast	-	-	-	7.23	812947	-
MIX WS	4.59	2235042	-	7.23	811766	-
Drug Product	4.59	2219663	99.31	7.23	810978	99.90

Every peaks were properly separated. When the chromatogram of the blank was examined at the retention time that corresponded to the peak of bilastine and montelukast, there were no peaks that interfered with the analysis. Both bilastine and montelukast were shown to have retention times of 4.59 minutes and 7.23 minutes, respectively, according to the observations made. The results of the test came out to be 99.90%.

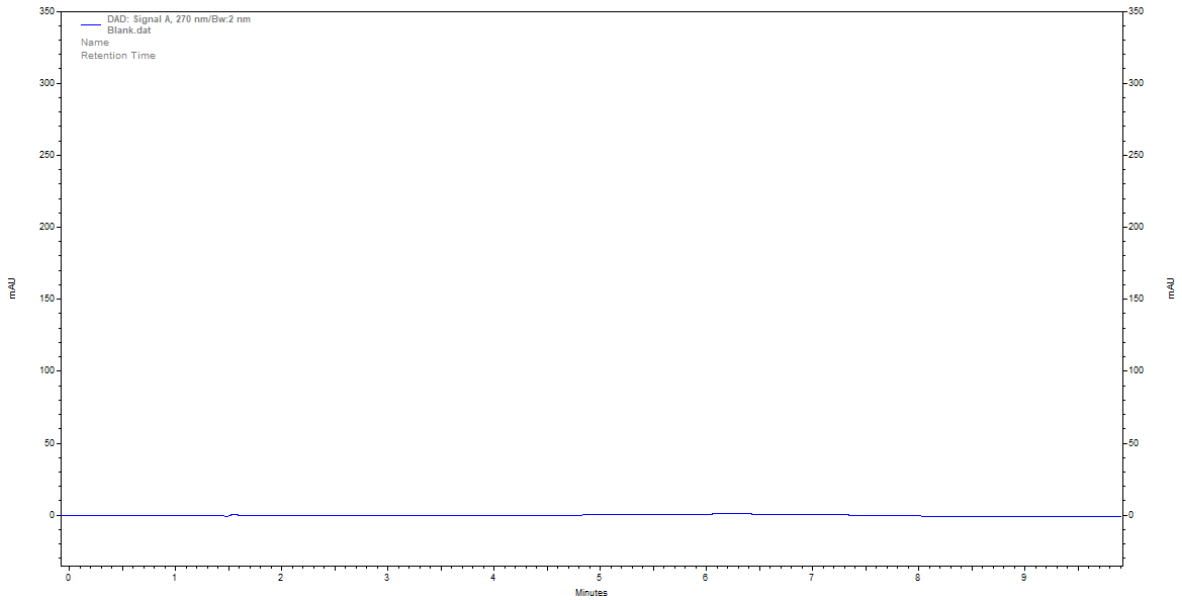


Figure 2: Specificity Blank-Diluent Chromatogram

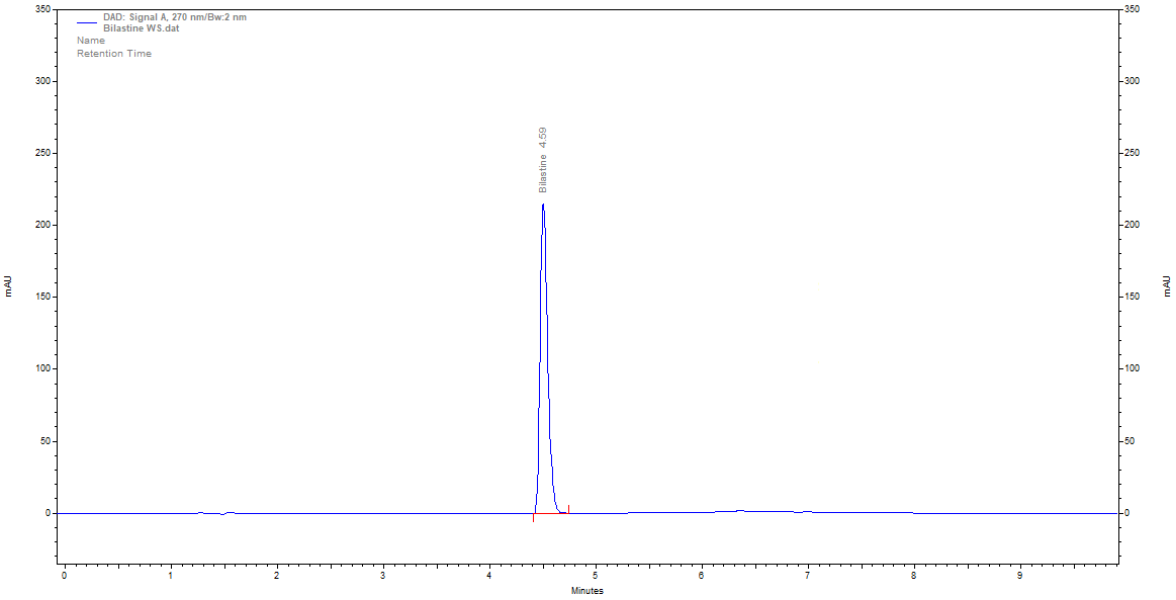


Figure 3: Bilastine Working Standard

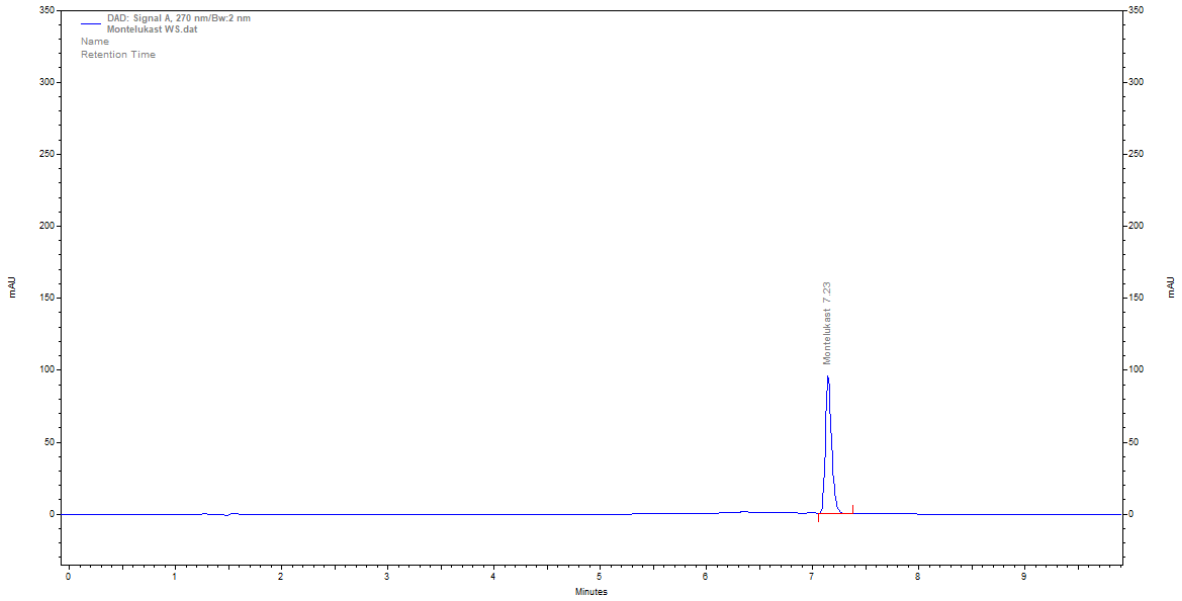


Figure 4: Montelukast Working Standard

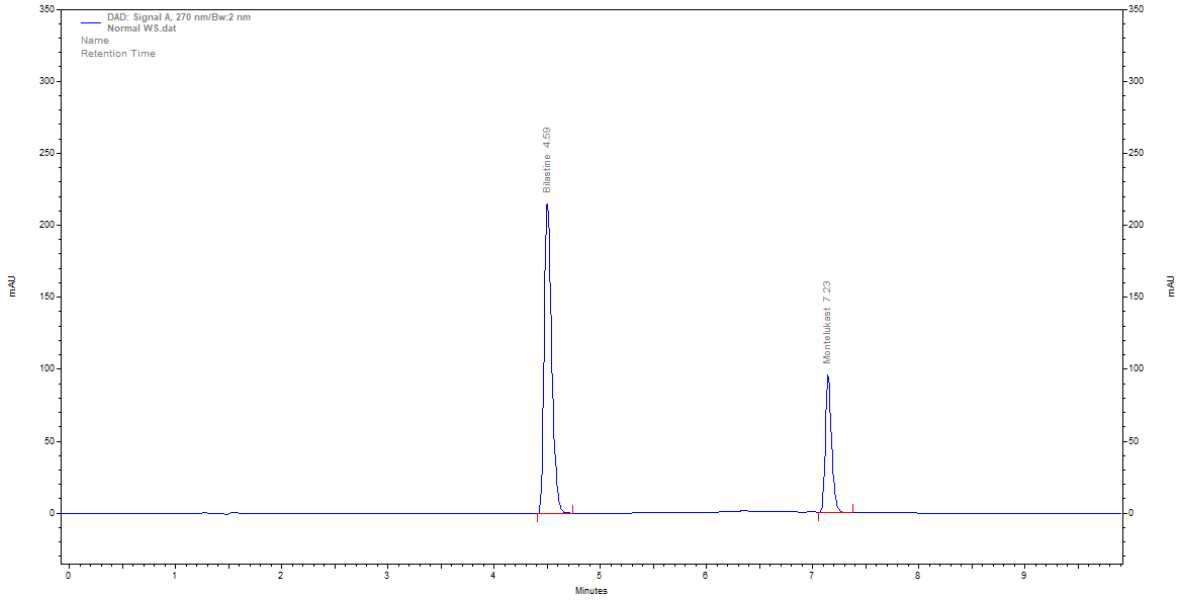


Figure 5: Mixture Working Standard

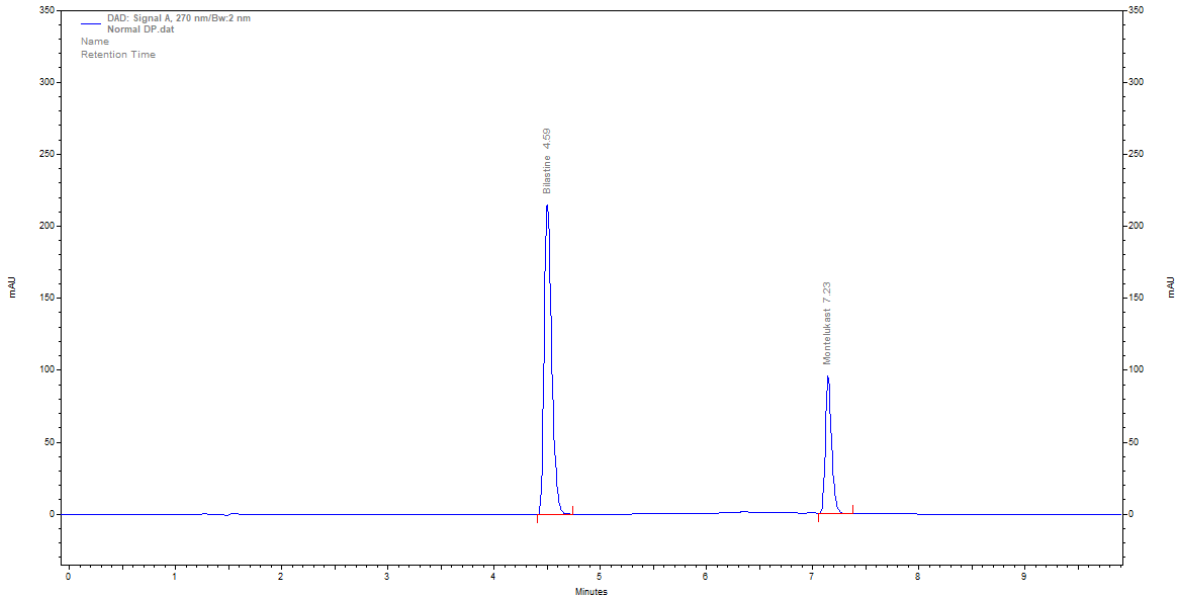


Figure 6: Drug Product

b. Instrument Precision and System suitability

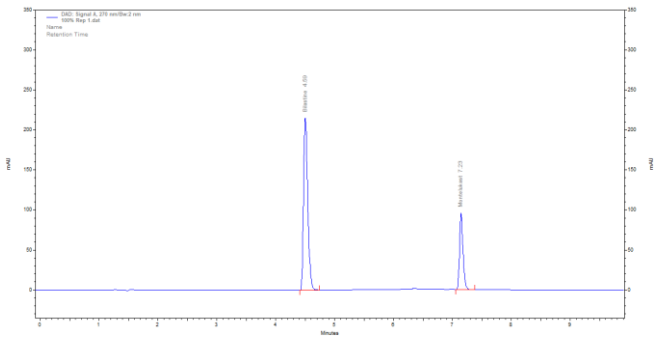
Instrument precision was performed for both APIs. The reported peak area is shown below:

Table 6: Instrument precision of Bilastine and Montelukast

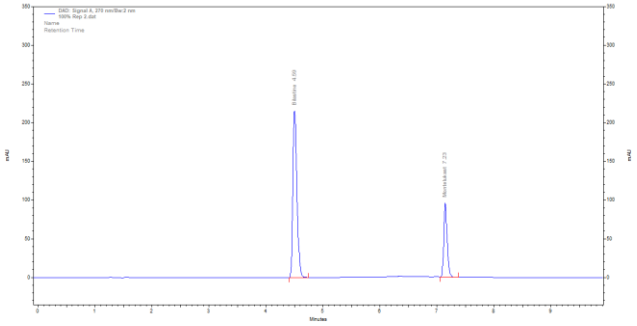
Repeatability		
	Peak Area	
Sample ID	**Bilastine**	**Montelukast**
100% Rep 1	2235042	811766
100% Rep 2	2217564	814854
100% Rep 3	2269745	813324
100% Rep 4	2225785	817452
100% Rep 5	2219754	815223
100% Rep 6	2254756	818523
AVG	2237108	815190
STDEV	20939.119	2511.7822
%RSD	0.94	0.31

From the above data, it can be seen that the %RSD for 6 replicate injections of Bilastine and Montelukast are 0.94% and 0.31% respectively. The %RSD is less than 2% as per the specification and guidance in ICH.

100% Rep 1



100% Rep 2



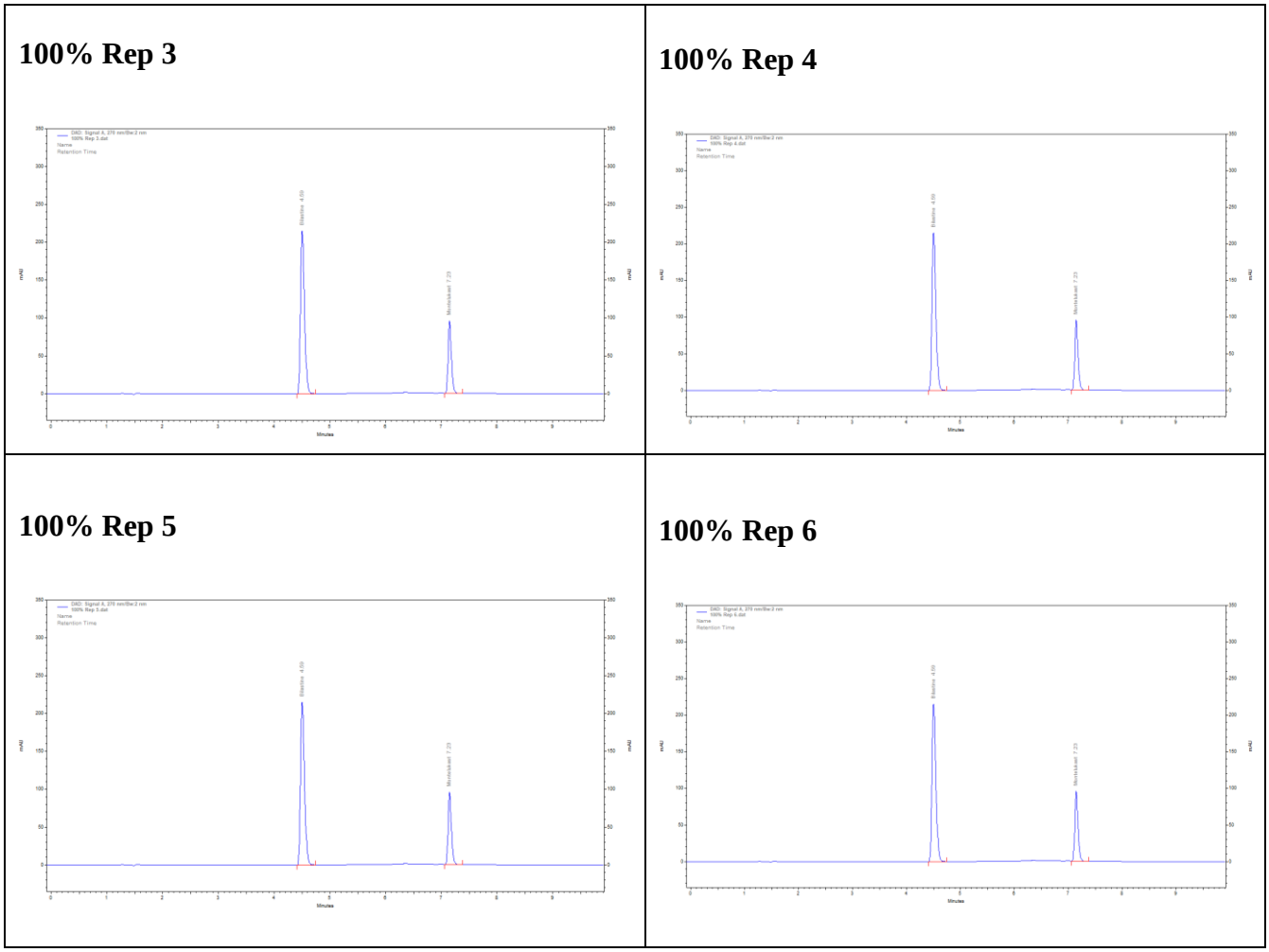


Figure 7: Instrument Precision of Bilastine and Montelukast

The system suitability criteria are per guidance ICH.

Criteria:

Theoretical Plates: more than 2000

Asymmetry: less than 2.0

Resolution: more than 2

%RSD of 6 replicates of working standard Retention time of main peak: less than 2%.

Table 7: System suitability for Bilastine

Bilastine				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	4.59	1.30	19918	0.00
Rep 2	4.59	1.31	21558	0.00
Rep 3	4.59	1.29	20459	0.00
Rep 4	4.59	1.27	19558	0.00
Rep 5	4.59	1.32	19333	0.00
Rep 6	4.59	1.28	21022	0.00
Avg	4.59			
STDEV	0.00			
RSD	0.00			

Table 8: System suitability for Montelukast

Montelukast				
Reps	**RT**	**Asymmetry**	**Theoretical Plates**	**Resolution**
Rep 1	7.23	1.36	75970	22.48
Rep 2	7.23	1.32	72895	22.48

d. LOD and LOQ

Based on the linearity data, LOD and LOQ was calculated and reported as below:

Table 9: LOD & LOD of Bilastine

Regression Statistics					
Multiple R	0.99996098				
R Square	0.999921962				
Adjusted R Square	0.99989595				
Standard Error	3618.358799				
Observations	5				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	5.03277E+11	5.03277E+11	38440.05293	2.92586E-07
Residual	3	39277561.2	13092520.4		
Total	4	5.03316E+11			
	Coefficients	*Standard Error*	*t Stat*	*P-value*	
Intercept	-6064.8	11556.11129	-0.524813222	0.636025601	
X Variable 1	22433.84	114.422552	196.0613499	2.92586E-07	

LOD	1.70	ug/ml
LOQ	5.15	ug/ml

Table 10: LOD & LOD of Montelukast

Regression Statistics					
Multiple R	0.99950887				
R Square	0.999017982				
Adjusted R Square	0.998690642				
Standard Error	4637.916745				
Observations	5				
ANOVA					
	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	65647913476	65647913476	3051.933248	1.30646E-05
Residual	3	64530815.2	21510271.73		
Total	4	65712444291			
	Coefficients	*Standard Error*	*t Stat*	*P-value*	
Intercept	-731.8	14812.31824	-0.049404826	0.963701941	
X Variable 1	16204.68	293.3276102	55.24430512	1.30646E-05	

LOD	3.02	ug/ml
LOQ	9.14	ug/ml

From the above data it was found that:

In the case of bilastine, the LOD and LOQ were discovered to equal 1.70 µg/ml and 5.15µg/ml.

According to the findings, the LOD and LOQ for Montelukast are as 3.02µg/ml and 9.14 µg/ml respectively.

e. Accuracy

Accuracy was performed at three different concentration levels. The % recovery and %RSD of 3 replicate injections were calculated. The acceptance criteria for replicate injection are that the %RSD should be less than 2%.

Table 11: Accuracy for Bilastine

Bilastine		
Std Wt. (mg)	**% Purity**	**Stock Conc. (ug/ml)**
10	99.7	997.00
Std Area	2237108	

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	79.76	1785845	79.59	99.79	99.69	0.360785	0.36
	Rep 2		1776987	79.19	99.29			
	Rep 3		1789554	79.75	99.99			
100%	Rep 1	99.70	2235042	99.61	99.91	100.16	1.187252	1.19
	Rep 2		2217564	98.83	99.13			
	Rep 3		2269745	101.15	101.46			
120%	Rep 1	119.64	2685465	119.68	100.03	99.87	0.256351	0.26
	Rep 2		2673112	119.13	99.57			
	Rep 3		2684545	119.64	100.00			

The %RSD of three replicates of Bilastine for accuracy level 80%, 100% and 120% was found to be 0.36%, 1.19% and 0.26% respectively.

The % recoveries for accuracy level 80%, 100% and 120% was found to be 99.69%, 100.16% and 99.87% respectively.

Table 12: Accuracy for Montelukast

Montelukast		
Std Wt. (mg)	**% Purity**	**Stock Conc. (ug/ml)**
5	99.7	498.50

Std Area	815190

Sample ID	Reps	Spiked Conc (ug/ml)	Area	Amount Recovered (ug/ml)	% Recovery	AVG	STDEV	%RSD
80%	Rep 1	39.88	646386	39.53	99.12	99.05	0.078609	0.08
	Rep 2		646122	39.51	99.08			
	Rep 3		645396	39.47	98.96			
100%	Rep 1	49.85	811766	49.64	99.58	99.77	0.189406	0.19
	Rep 2		814854	49.83	99.96			
	Rep 3		813324	49.74	99.77			
120%	Rep 1	59.82	974817	59.61	99.65	99.47	0.159799	0.16
	Rep 2		971985	59.44	99.36			
	Rep 3		972254	59.45	99.39			

The %RSD of three replicates of Montelukast for accuracy level 80%, 100% and 120% was found to be 0.08%, 0.19% and 0.16% respectively.

The % recoveries for accuracy level 80%, 100% and 120% was found to be 99.05%, 99.77% and 99.47% respectively.

The chromatograms for accuracy are given below:

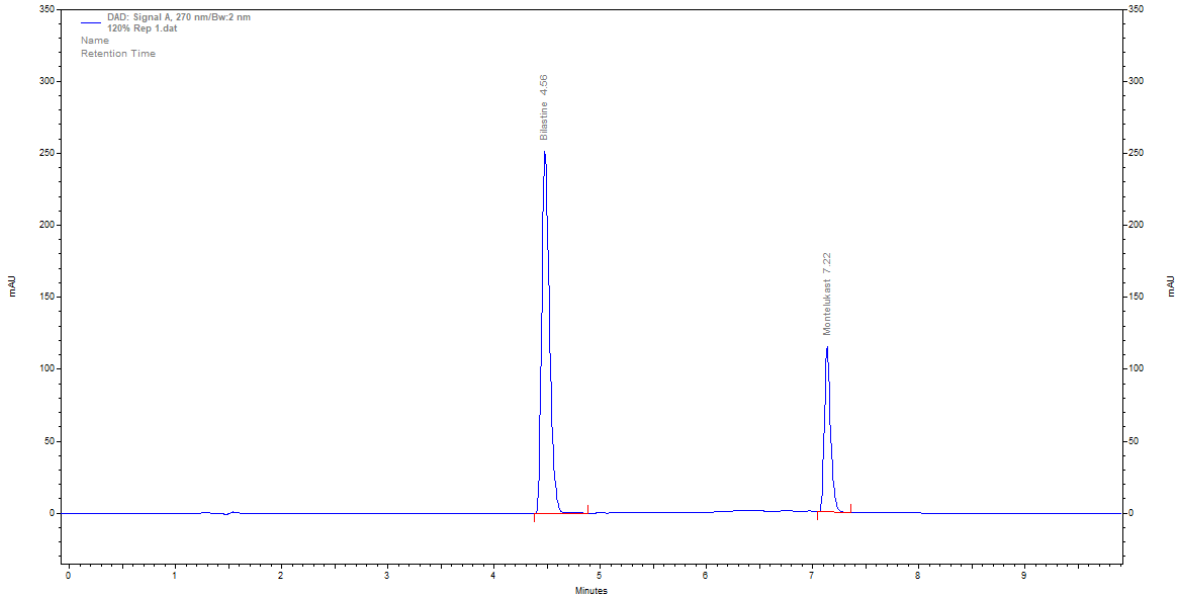


Figure 8: Accuracy Level- 120% Rep 1

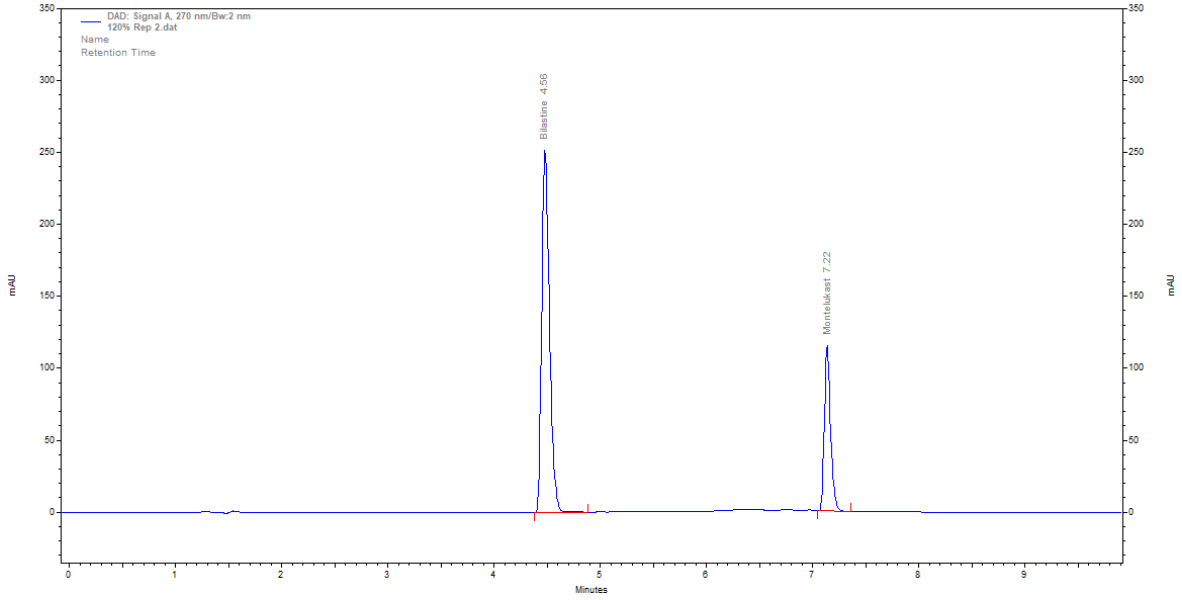


Figure 9: Accuracy Level- 120% Rep 2

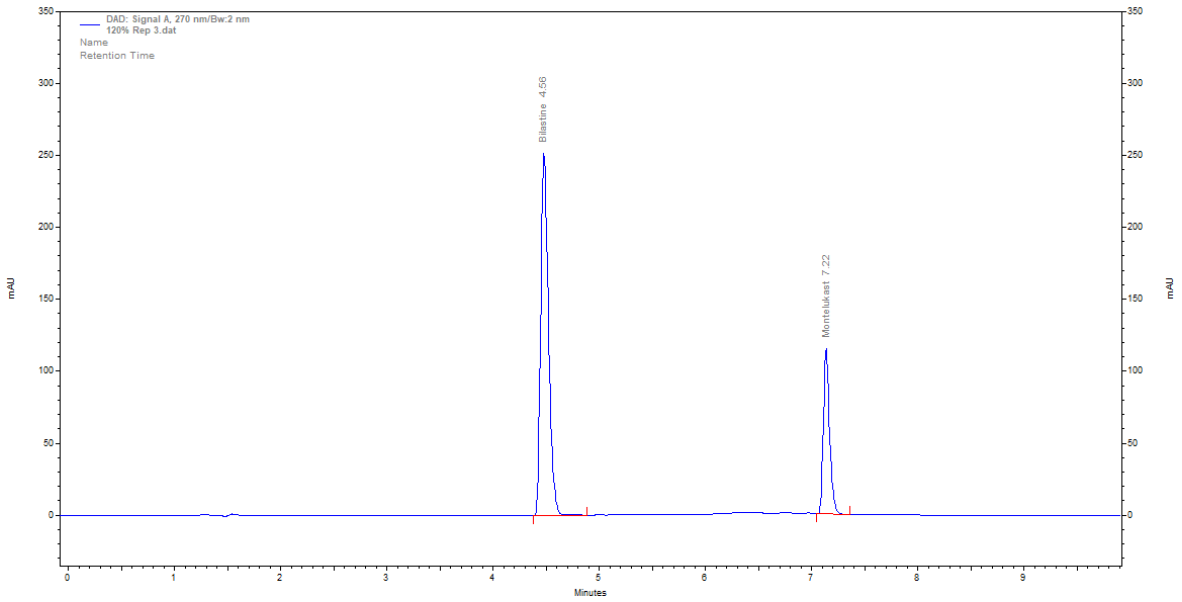


Figure 10: Accuracy Level- 120% Rep 3

f. Robustness Study

Robustness study was done change in column temperature and change in wavelength.

Table 13: Robustness study - Change in Column temperature

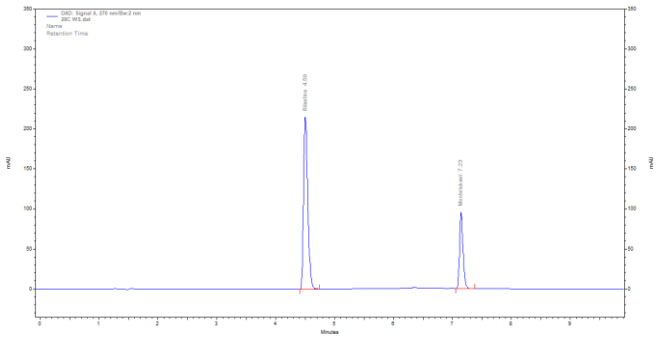
Column Oven Temp Change					
Condition	**Sample**	**Bilastine**		**Montelukast**	
		Peak Area	**%Assay**	**Peak Area**	**%Assay**
28°C	**WS**	2219458	-	811337	-
	DP	2216554	99.87	809222	99.74
30°C	**WS**	2235042	-	811766	-
	DP	2219663	99.31	810978	99.90
32°C	**WS**	2234268	-	809445	-
	DP	2230199	99.82	808499	99.88

The Assay of Bilastine at 28ºC, 30 ºC and 32 ºC was found to be 99.87%, 99.31% and 98.82% respectively.

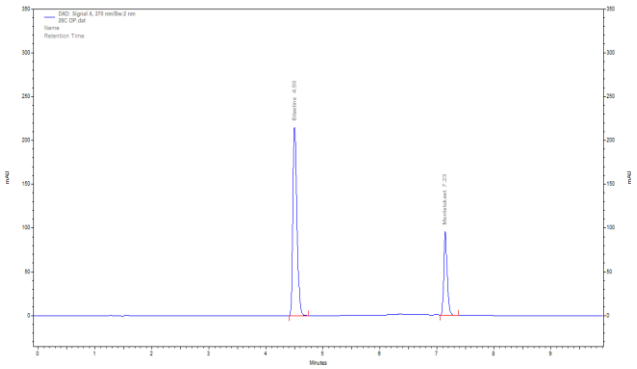
The Assay of Montelukast at 28ºC, 30 ºC and 32 ºC was found to be 99.74%, 99.90% and 99.88% respectively.

The chromatograms are given below:

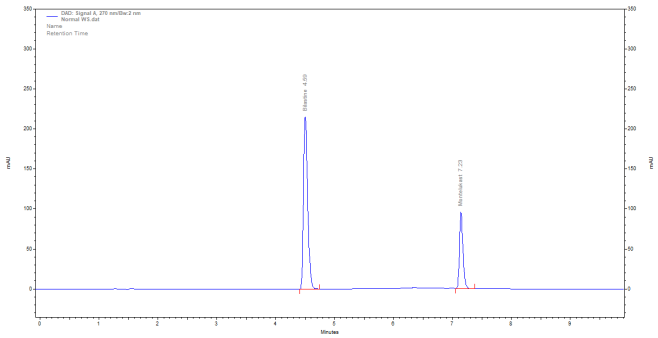
28˚C Working Standard



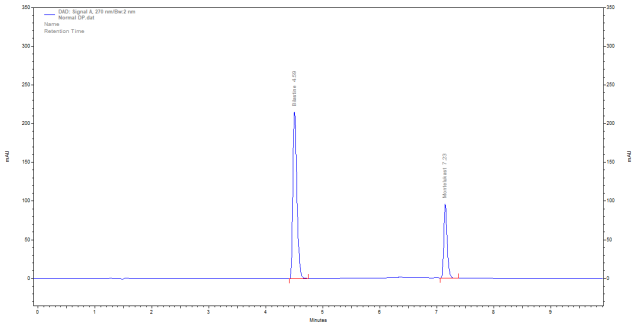
28˚C Drug Product



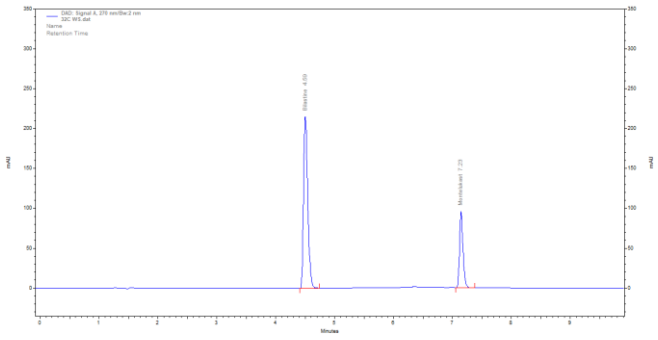
30˚C Working Standard



30˚C Drug Product



32˚C Working Standard



32˚C Drug Product

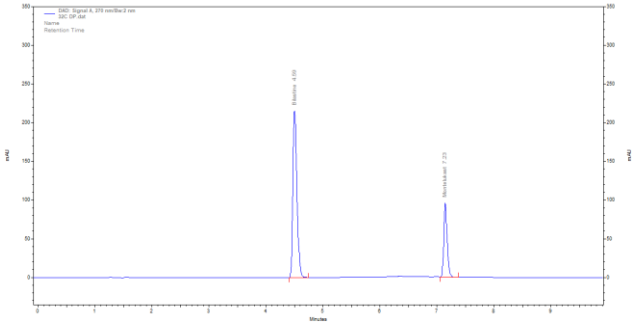


Figure 11: Robustness Study of Bilastine and Montelukast (Change in Column Oven Temperature)

Table 14: Robustness study: Change in wavelength

Wavelength (nm)					
Condition	**Sample**	**Bilastine**		**Montelukast**	
		Area	**Assay**	**Area**	**Assay**
268	**WS**	2198642	-	810333	-
	DP	2185549	99.40	809478	99.89
270	**WS**	2235042	-	811766	-
	DP	2219663	99.31	810978	99.90
272	**WS**	2201237	-	812795	-
	DP	2194468	99.69	811497	99.84

The Assay of Bilastine at 268 nm, 270 nm and 272 nm was found to be 99.40%, 99.31% and 99.69% respectively.

The Assay of Montelukast at 268 nm, 270 nm and 272 nm was found to be 99.89%, 99.90% and 99.84% respectively.

The chromatograms are given below:

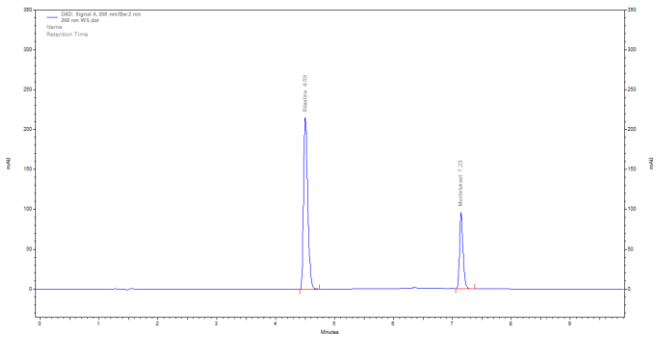
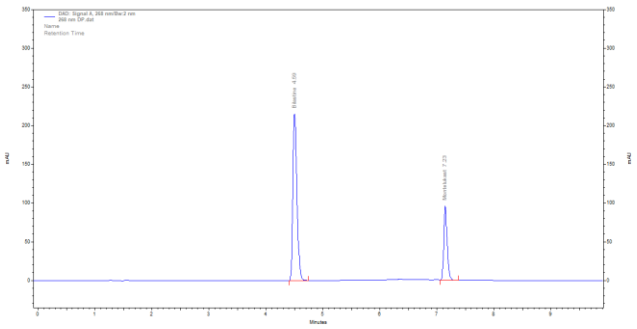
268 nm Working Standard	**268 nm Drug Product**
	

Figure 12: Robustness Study of Bilastine and Montelukast (Change in Wavelength)

As a result, it was discovered that the approach was reliable even when subjected to a slight variation in the column temperature and the wavelength. There was no discernible change in either the retention time or the area of the replicate injection process.

g. Intra and Inter day precision

An intra-day and inter-day precision investigation was carried out, and the percentage relative standard deviation (RSD) change in peak area of the APIs at various time intervals was reported. The relative standard deviation (RSD) of the peak area must be less than 2% in order to meet the acceptance standards.

Table 15: Intra & Interday Precision

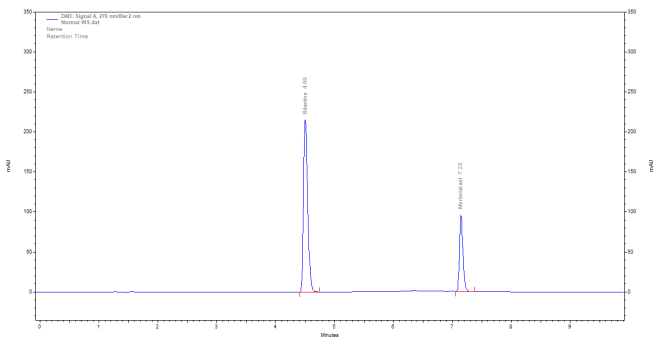
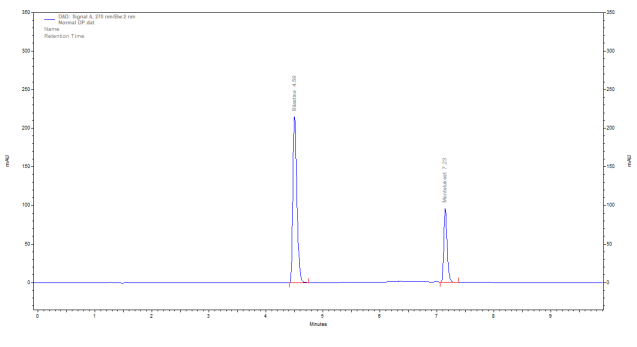
Intra Day precision					
Day 1	**Sample ID**	**Bilastine**		**Montelukast**	
		Area	**Assay**	**Area**	**Assay**
Morning	**WS**	2235042	-	811766	-
	DP	2219663	99.31	810978	99.90
Evening	**WS**	2197562	-	812899	-
	DP	2185645	99.46	810548	99.71
Inter Day precision					
Day	**Sample ID**	**Bilastine**		**Montelukast**	
		Area	**Assay**	**Area**	**Assay**
Day 2	**WS**	2207899	-	810545	-
	DP	2198311	99.57	809321	99.85
%RSD		0.13		0.10	

The Assay of Bilastine at morning, evening and Day 2 was found to be 99.31%, 99.46% and 99.57% respectively.

The Assay of Montelukast at morning, evening and Day 2 was found to be 99.90%, 99.71% and 99.85% respectively.

Bilastine and Montelukast were shown to have a relative standard deviation (RSD) of 0.13% and 0.10%, respectively, for intra and inter-day precision, respectively.

The chromatograms for intra and inter day precision are given below:

Morning Working Standard	**Morning Drug Product**
	

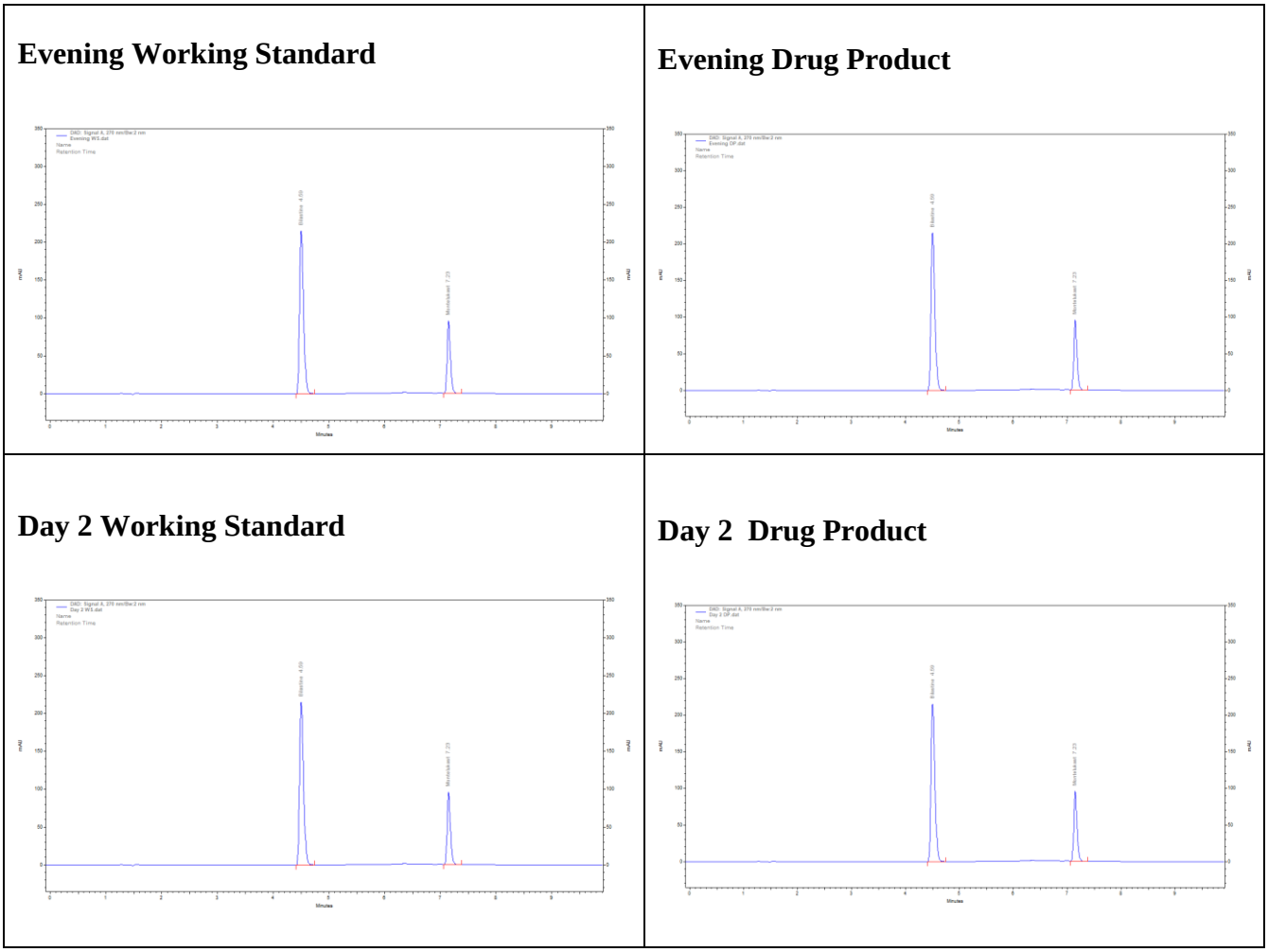


Figure 13: Intra & Inter Day precision of Bilastine and Montelukast

There is no change in the stability of the working standard solution of bilastine and montelukast after two days, according to the percentage assay of the APIs peak.

CONCLUSION:

The purpose of this study was to develop and validate a RP-HPLC method for the quantification of bilastine and montelukast in tablet formulations and bulk quantities. The proposed methods were found to be appropriate due to its simplicity, reliability, sensitivity, rapidness and selectivity for detection at very low concentrations. The short chromatographic time makes this method suitable for processing of multiple samples in short time. The method showed no interference of the Excipients present in Bilastine and Montelukast. The statistical parameters and recovery data reveals the good accuracy and precision of the proposed methods. The RP-HPLC method developed for the estimation of Bilastine and Montelukast was validated as per the ICH guidelines.

Validation data demonstrates that, these methods are accurate, precise, simple and economic and can be used in the routine analysis of Bilastine and Montelukast in various formulations.

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