SIMULTANEOUS ESTIMATION OF BILASTINE AND MONTELUKAST IN BULK BY RP-HPLC AND ASSESSMENT OF ITS APPLICABILITY IN MARKETED TABLET DOSAGE FORM

ABSTRACT

~~Aims~~ Aim: This study proposes to develop and validate the RP-HPLC method for Bilastine and Montelukast and to substantiate the RP-HPLC analysis bestowing to ICH validation guideline Q2R1.

Place and Duration of Study: Y. B. Chavan College of Pharmacy, Aurangabad, MS, India, between January 2020 and October 2021.

Methodology: The mixture of drugs was subjected to optimization by trial runs with different chromatographic parameters, viz. flow rate, λ in nm, etc. The system suitability was performed by repeated injections of Bilastine (200µg/ml) and Montelukast (200µg/ml) to confirm the optimization. Furthermore, the demonstrated method was validated as per ICH Q2R1 recommendations for parameters like accuracy, precision, robustness, the limit of detection and quantitation, etc.

Results: The outcomes of the method in terms of percent relative standard deviation (%RSD) of retention time (RT) and mean peak area were seen as 0.09, 0.35, and 0.35, 0.56 for Bilastine and Montelukast, respectively. The method was successful in achieving the qualifying criteria entrusted ~~in~~ to ICH guidelines. The correlation coefficient, slope, and y-intercept were illustrated to be 0.9971, 17595, 217883, and 0.998, 35458, and 17147, correspondingly for Bilastine and Montelukast, respectively. The range was seen in the order of 160-260µg.ml and 80-130µg/ml for Bilastine and Montelukast. The precision of the method was established with %RSD of repeatability and intermediate precision < 2 at three standard levels across the range. The %accuracy of the method was observed in the range of 96.95-101.41 %w/w and 97.37-101.89 %w/w in the order for Bilastine and Montelukast. The robustness of the method displayed the results within the prescribed boundaries. The recovered amount of Bilastine and Montelukast by spike method was observed to be 96.37-98.88 %w/w and 96.11-100.06%w/w.

Conclusion: The author has accomplished the predefined goals by successful development and validation of the RP-HPLC method for the quantification of Bilastine and Montelukast as per ICH Q2R1 guidelines.

Keywords: Bilastine, Montelukast, RP-HPLC, Precision, Linearity and Range, %Accuracy, Robustness.

Formatted: Underline

Formatted: Underline

Formatted: Underline

Formatted: Underline

1. INTRODUCTION

The efficiency and speed of High-Performance Liquid Chromatography (HPLC) have proved the method's development requirements in the past 30 years [1, 2]. Recently, the HPLC has proved to be the most valuable technique for a custom analysis of peptides [3, 4]. Hence, HPLC has been found to be the first choice of analytical scientists for qualitative and quantitative analysis of drug substances and drug products. Further, HPLC is competent enough to separate the most complex mixtures [5,6].

Bilastine (BIL) chemically is 2-[4-(2-{4-[1-(2-ethoxyethyl)-1H-1,3-benzodiazol-2-yl]piperidin-1-yl}ethyl)phenyl]-2-methylpropanoic acid. It works by acting on H-1 histamine receptors. It is recommended for patients suffering from allergic rhinitis and chronic urticaria [7,8]. It is available in the dosage of 10-20mg alone or combination with Montelukast [9]. The structure of Bilastine is shown in figure 1.

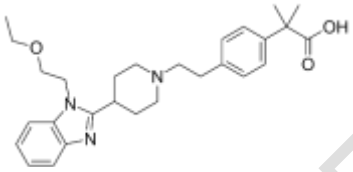

Fig. 1. The Structure of Bilastine

Montelukast (MTL) chemically is 2-[1-({[(1R)-1-{3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl}-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanyl}methyl)cyclopropyl]acetic acid. It is a leukotriene receptor antagonist of Cysteinyl Leukotriene (CysLT) type 1 receptor. After binding to CysLT type 1 receptor, bilastine causes inhibition to physiological effects of CysLT's like LTC4, LTD4, and LTE4 [10]. The outcome of this inhibition is the prevention of the symptoms of allergic rhinitis and asthma [11, 12]. The structure of MTL is as shown in figure 2.

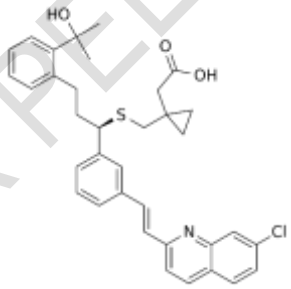

Fig. 2. Structure of Montelukast

Literature research found chromatographic, and spectroscopic analysis of Bilastine, Montelukast either alone or in combination with other drug substances. No reports were found in the literature ~~which~~ that explore the quantification of Bilastine and Montelukast in a mixture using reverse-phase HPLC. ~~As the HPLC is the most acceptable tool of analysis of drug substances and drug products, there is an unmet need to develop and validate the RP-HPLC method for quantification of Bilastine and Montelukast in a mixture using RP-HPLC.~~ Because HPLC is the most widely used method for analyzing drug substances and drug products, there is an unmet need to develop and validate the RP-HPLC method for quantifying bilastine and montelukast in a mixture using RP-HPLC. Hence, this research work was undertaken.

2. MATERIAL AND METHODS

2.1 Materials

Potassium dihydrogen phosphate, tri-ethylamine, and orthophosphoric acid were purchased from a local chemical distributor ~~of make~~ by Thermo Fisher Scientific India. The Bilastine and Montelukast were purchased from a local vendor ~~at~~ in Aurangabad, MS, India. The Bilasure M tablet (Hetero Labs Ltd.) with the strength of 20mg Bilastine and 10mg Montelukast was procured from a local medical store in the aforesaid city. This formulation was used to recover the amount of Bilastine and Montelukast from the pharmaceutical tablet dosage form to ensure the applicability of the method for a custom analysis of the fixed-dose combination.

2.2. Instrumentation

The HPLC (ThermoFisher) instrument was equipped with column C_{18} (250mm×4.6mm).

2.2 Methods/Experimental Work

2.2.1 Preparation of standard stock and working solution of the mixture.

20mg of Bilastine and 10 mg of Montelukast and transferred to the same 100ml volumetric flask containing a mixture of Acetonitrile: Phosphate buffer (pH 6.8) (60:40). The volume was made up to the mark with the help of the mobile phase. The consequential stock solutions of Bilastine (200µg/ml) and Montelukast (100µg/ml) were filtered through a 0.45µ membrane filter and ultrasonicated for three cycles each of 10 min. The solution of 200ppm of Bilastine and 100ppm of Montelukast was injected into a given set of chromatographic conditions to observe the response. Initially, the chromatographic conditions were varied as per the response observed after each injection. The details of the protocol that followed were as illustrated in table 1.

Table 1. Chromatographic conditions were tested while optimization of analysis of the mixture of BIL and MTL using RP-HPLC.

Exp No.	Flow (ml/Min)	Inj. Vol.	Column	Mobile Phase	Reason
1	1.0	10	Water Symmetry C18 250*4.6 5u	Water: Acetonitrile (50:50)	One peak is Not detected
2	1.0	10	Water Symmetry C18 250*4.6 5u	Water: Acetonitrile (10:90)	Symmetry is not up to the mark
3	1.0	10	Water Symmetry C18 250*4.6 5u	0.01 M Amm. Di hydro. Phosphate (pH-4.0): Acetonitrile(50:50)	Fronting is observed in second peak
4	1.0	10	Water Symmetry C18 150*4.6 5u	0.01 M Amm. Di hydro. Phosphate (pH-4.0): Acetonitrile(30:70)	Both peaks are close to each other

5	1.0	10	Luna C18 150*4.6 3u	0.01 M Amm. Di hydro. Phosphate (pH-4.0): methanol (50:50)	Symmetry is not up to the mark
6	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous (pH-6.8 with H3PO4):Acetonitrile (50:50)	Fronting is observed in second peak
7	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethylamine in 100 ml (pH-6.8 with H3PO4): Acetonitrile (50:50)	Symmetry is not up to the mark
8	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (50:50)	Inject Blank
9	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (45:55)	Symmetry is not up to the mark
10	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (45:55)	Symmetry is not up to the mark
11	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (40:60)	Inject bilastine for RT Detection
12	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (40:60)	Inject sample for RT Detection
13	0.6	10	Optimapac C8	0.01 M Di-sodium hydrogen phosphate	Inject

			(150*4.6 5u)	anhydrous +1 ml triethyl amine in 100 ml (pH-6.8 with H3PO4):Acetonitrile (40:60)	Montelukast for RT detection
14	0.6	10	Optimapac C8 (150*4.6 5u)	0.01 M Di-sodium hydrogen phosphate anhydrous +1 ml triethylamine in 100 ml (pH-6.8 with H3PO4): Acetonitrile (40:60)	

2.2.2 System suitability testing

The identical standard solution of Bilastine 200µg/ml and Montelukast 100µg/ml in the blend was injected in the RP-HPLC column with succeeding (table 2) optimized chromatographic parameters and the chromatogram was recorded. The chromatogram was analyzed to estimate retention time, peak area, number of theoretical plates, tailing factor, etc. The obtained results were compared with limits given in ICH guidelines Q2R1. The correspondent procedure was adopted an added five times and outcomes were noted in each case of the chromatogram seen. The mean retention time and mean area were calculated accordingly.

Table 2. Optimized chromatographic conditions

Chromatographic Conditions	
Column	C_{18} (250mm×4.6mm), 5µm id
Mobile phase	Acetonitrile 60: Disodium hydrogen Phosphate buffer 40 (pH 6.8) v/v
Detection Wavelength	254 nm (Isobestic Point)
Flow rate	0.6 mL/min
Temperature	25 ºC
Sample size	10µl
Run Time	15 minutes

2.2.3 Method validation

2.2.3.1 Linearity and Range

Aliquots of 0.8, 0.9, 1.0, 1.1, 1.2, and 1.3ml standard stock solution (Bilastine 200µg/ml and Montelukast 100µg/ml) were pipette out and taken ~~in to~~ into10ml volumetric flask. The volume of the latter was made up to 10ml with mobile (Acetonitrile 60: Phosphate buffer 40, pH 6.8) to get working solutions of 160, 180, 200, 220, 240, 260µg/ml for Bilastine and 80, 90, 100, 110, 120 and 130µg/ml for Montelukast. All of these standard working solutions of Bilastine and Bilastine (in the mixture) were injected as a mixture in triplicate ~~to~~ into the optimized chromatographic conditions and mean peak areas were determined. The calibration curve was constructed ~~among~~ using the concentration of standard solutions of Bilastine and Montelukast. The mean peak area was estimated to be consequential for each chromatographic measurement. From the calibration curve equation of the line, the correlation coefficient, and intercept were estimated. The general equation of a straight line is as depicted below.

$$Y = mX + c$$

Where, Y = Peak area; m = slope; X = measured concentration; c = intercept.

2.2.3.2 Precision

Three standard solutions of the mixture of Bilastine and Montelukast were used across the given range (160 to 260µg/ml of Bilastine and 80-130 µg/ml of Montelukast to establish the precision of the method. The repeatability was recognized by repeated measurements of standard solutions on the same day. However, intermediate precision (system precision) was established on different days in the series. The three standards, viz., 170, 210, and 250µg/ml for Bilastine and 85, 105, and 125µg/ml for Montelukast were injected, and the mean peak area was integrated from the chromatograms. The %RSD was calculated in each case and compared with the prescribed standards for its compliance.

2.2.3.3 %Accuracy

The accuracy of the method was established by using three standard solutions of ~~the~~ Bilastine and Montelukast in the mixture (API mixture) as cited in the precision study. The solutions were injected in triplicate and the corresponding concentration was estimated by extrapolation on the calibration curve. The %accuracy was then estimated using the following formula~~.~~ :

$$\% \text{ Accuracy} = \frac{\text{Mean measured concentration}}{\text{Nominal Concentration}} \text{ x } 100$$

2.2.3.4 Robustness

The robustness of the method was studied by deliberate variations in the three method parameters, viz., organic concentration of the mobile phase, mobile phase flow rate in 'mL/min', and detector wavelength in 'nm'. The study design was as prescribed in table 3.

Table 3. The study design in the robustness experiment shows the actual variation in the method parameter.

Method parameter	Standard	Variation 1	Variation 2
Wavelength in 'nm'	254	250	258
Flow rate of mobile phase in mL/min (± 0.15ml/min)	0.6	0.45	0.75
Organic conc. of Mobile phase (± 5%)	60	55	65

Concentrations of Bilastine (200µg/ml) and Montelukast (100µg/ml) were injected as a mixture of the solution to previously optimize chromatographic situations in triplicate at each level of change, and chromatograms observed were noted. From the chromatograms that resulted, the mean peak area was calculated. The %RSD was then calculated and assessed for its compliance as per ICH guidelines.

2.2.3.5 %recovery studies and assessment of the applicability of the method for a custom analysis of Bilastine and Montelukast as Fixed-Dose Combination (FDC) in marketed tablet dosage form.

Preparation of stock from API

Formatted: Font: Bold

They ~~Accurately~~ accurately weighed 20mg of Bilastine and 10mg of Montelukast (API) and were transferred ~~in~~ to an identical 100 ml volumetric flask having an indistinct quantity of mobile phase. The volume of the later was made up to the mark with the mobile phase to attain a concentration of 200µg/ml for Bilastine and 100 µg/ml for Montelukast. The resulting solution was filtered through a 0.45µ membrane filter and ultra-sonicated for three cycles each of 10 min. The same procedure was repeated twice to prepare another two solutions with identical concentrations in two 100ml volumetric flasks. These (three) solutions were injected ~~in~~ into a fitting chromatographic system in triplicate, and the mean peak area in each case was estimated. The peak area was noted ~~in~~ and kept aside.

Preparation of stock from the dosage form

Formatted: Font: Bold

Twenty tablets of the combined dosage form of Bilastine and Montelukast (Bilasure M 20/10, Label claim Bilastine 20mg, Montelukast 10mg, Hetero Labs Ltd.) were weighed; average weight (0.3187gm) was determined and powdered. A ~~Powder~~ powder equivalent to 20mg of Bilastine, 0.3187g (10mg of Montelukast) was weighed and taken to a 100ml volumetric flask with the approximate amount of mobile phase. The volume was made up to the mark with consequential shaking to achieve the main stock sample solution of Bilastine 200µg/ml (100µg/ml for Montelukast). The ensuing sample solution was filtered through a 0.45µ membrane filter and ultra-sonicated for three cycles each of 10 min. Aliquots of 0.8 and 1.2ml were pipette out from the sample stock solution (200µg/ml and 100µg/ml) to attain the ready test solutions of 160 and 240µg/ml (80 ~~&~~ and 120µg/ml for Bilastine and Montelukast respectively). The three sample solutions of combined dosage form viz. 160, 200 & 240µg/ml and 80, 100, and 120µg/ml (Bilastine and Montelukast respectively) were labeled as three levels of percent recovery testing viz. 80, 100, and 120% in that order.

Preparation of test solution for % recovery by spike method

Formatted: Font: Bold

Three 200µg/ml & 100µg/ml standard solutions of the mixture (Bilastine and Montelukast) (API) were spiked into every sample solution of the combined dosage form viz. 160, 200 & 240µg/ml and 80, 100 and 120µg/ml to attain test solutions at 80%, 100% and 120% levels likewise. ~~Each of these three percent recovery levels was injected in triplicate in previously optimized chromatographic conditions of the projected method.~~ Each of these three percent recovery levels was injected in triplicate under previously optimized chromatographic conditions of the proposed method. The mean peak area for each percent recovery level was determined. The mean peak area obtained on API injection (formerly estimated) was subtracted from the mean peak area of each of these three percent recovery levels to get the peak area corresponding to each sample solution. The recovered amount of Bilastine and Montelukast was calculated from the test concentration and standard concentrations and their equivalent mean peak area using the following ~~subsequent~~ formula.

$$\% \text{ Recovery} = \frac{Sample\ Peak\ Area}{Standard\ Peak\ Area} \times \frac{Standard\ Concentration}{Sample\ Concentration} \times 100$$

2.2.3.6 LOD and LOQ

Limit of detection (LOD) and Limit of quantitation (LOQ) for Bilastine and Montelukast was calculated from the resultant formulae.

$$LOD = \frac{3.3 * STEYX}{Slope}$$

$$LOQ = \frac{10 * STEYX}{Slope}$$

Where STEYX = Standard error of Y and X-axis.

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 System suitability testing

The study was performed with six repeated measurements of BIL and MTL in the mixture at 100% concentration, viz., 200µg/ml & 100µg/ml for BIL and MTL, respectively. The chromatograms observed were integrated to determine peak area, standard deviation (SD), and %RSD. The results observed ~~were~~ are tabulated in table 4 for BIL and table 5 for MTL. The representative chromatogram of the study ~~was as~~ is shown in figure 3.

The other parameters (as listed below) were compared for their compliance as per ICH guideline Q2R1.

- Several theoretical plates or Efficiency (N).
- Capacity factor (K).
- Separation or Relative retention (α).
- Resolution (Rs).
- Tailing factor (T).
- Relative Standard Deviation (RSD).

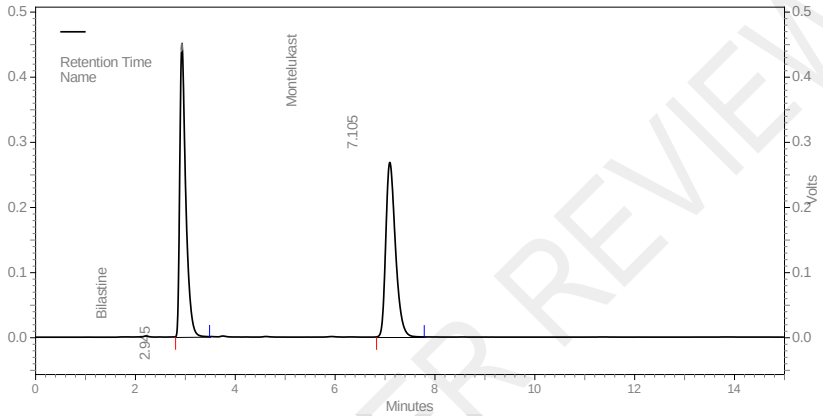

Fig. 3. The chromatogram was observed for quantification of a mixture of BIL and MTL showing retention time at 2.945 and 7.105 min. respectively.

Table 4. The observations noted for BIL in the system suitability testing experiment

Sr. No.	Parameter	Mean observations	SD	%RSD	Acceptance criteria	Inference
1	Peak Area	3764605.67	20030.83	0.53	< 2	Complied
2	Retention time	2.95	0.00	0.09	< 0.5	Complied
3	Number of Theoretical plates (NOP)	3118	--		> 2000	Complied
4	Tailing factor	1.69	--		< 2	Complied

The %RSD observed for BIL for RT and mean peak area were 0.09 and 0.53 respectively. Whereas, the %RSD of MTL for RT and mean peak area were 0.35 and 0.56 respectively. The NOP of BIL and MTL were3118 and 6567 respectively. The asymmetry factor was observed to be 1.69 and 1.38 in that order.

Table 5. The observations noted for MTL in the system suitability testing experiment

Sr. No.	Parameter	Mean observations	SD	%RSD	Acceptance criteria	Inference
1	Peak Area	3603723.17	20306.80	0.56	< 2	Complied
2	Retention time	7.13	0.03	0.35	< 0.5	Complied
3	Number of Theoretical plates	6567	--		> 2000	Complied
4	Tailing factor	1.38	--		< 2	Complied

3.1.2 Method validation

3.1.2.1 Linearity and range

The linearity of the method was assessed by injecting a series of standard solutions of BIL (160-260µg/ml) and MTL (80-130µg/ml) into the mixture. The mean peak area was integrated from the chromatogram observed. The peak area corresponding to each standard solution of BIL and MTL was as illustrated in table 6.

Table 6. Observed mean peak area corresponding to each standard concentration of BIL & MTL

Sr. No.	Conc. of BIL std. solution (µg/ml)	Mean peak Area*	Conc. of MTL std. solution (µg/ml)	Mean peak Area*
1	160	3042996	80	2845902
2	180	3408528	90	3176105
3	200	3668182	100	3471319
4	220	4109927	110	3897564
5	240	4461306	120	4241004
6	260	4786308	130	4603775

*mean peak area of three repeated measurements; BIL: Bilastine, MTL: Montelukast

The calibration curve was plotted from the mean peak area and standard concentrations of BIL ~~&~~ and MTL. The calibration curve obtained was as shown in Figures 4 ~~&~~ and 5 for BIL and MTL respectively. From the calibration curve, the equation of the line, slope, and *y*-intercept were calculated. The further regression coefficient was estimated in each case and found to be 0.9971 and 0.998 for BIL and MTL, respectively.

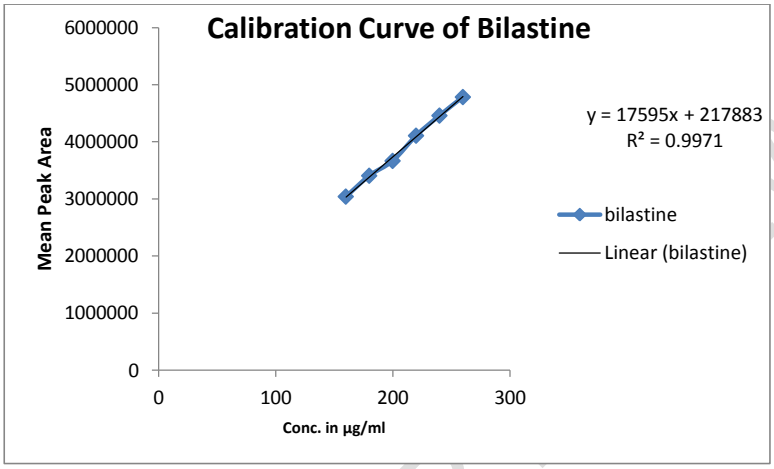

Fig. 4. Calibration curve of Bilastiine

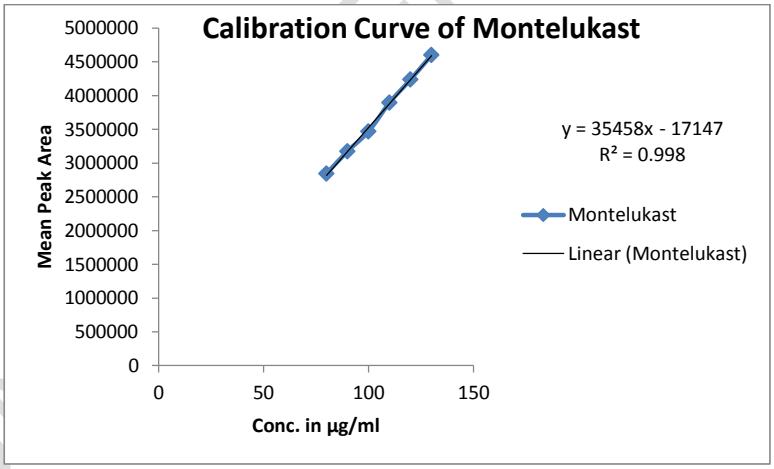

Fig. 5. Calibration curve of Montelukast

The equation of line was observed to be y = 17595*x* + 217883 and y = 35458*x* – 17147 for BIL and MTL respectively.

3.1.2.2 Precision

The precision experiment was performed in two ways.

- Repeatability: precision under interchangeable working conditions, a similar analyst over a quick period.
- Intermediate precision: the system is assessed on an array of days.

The ICH guidelines advocate that repeatability should be documented appropriately using a minimum of nine determinations through the standard range for the method (e.g., three concentrations / three replicates each) or a minimum of six repeated measurements at 100% of the assessment concentration. In this case, it was performed by the prior method.

The three standard solutions at three levels across the range of the method of the BIL and MTL in the mixture were injected and chromatograms were recorded. The peak area was integrated and subjected to statistical analysis to determine ~~to~~ the mean peak area, SD, and %RSD. The results of the %RSD observed in the repeatability study of BIL were in the range of 0.44-1.97. The %RSD for intermediate precision was in the range of 1.05 to 1.68 (table 7). The outcomes are seen within the boundaries prescribed.

Table 7. The repeatability and intermediate precision outcomes of the BIL

Conc. (µg/ml)	Intra-day precision (Repeatability)			Inter-day precision (Intermediate precision)		
	Mean area ± SD	**% RSD**	**Inference**	**Mean area ± SD**	**% RSD**	**Inference**
170	4396457.33 ± 19306.48	0.44	Complied	3251179.00 ± 54586.81	1.68	Complied
210	3284226.00 ± 44307.19	1.35	Complied	3800053.33 ± 47890.94	1.29	Complied
250	4551229.67 ± 89524.67	1.97	Complied	4557073.33 ± 47890.94	1.05	Complied

The results of the %RSD observed in the repeatability study of MTL were in the range of 0.44-1.95. The %RSD for intermediate precision was in the range of 0.44 to 1.97 (table 8). The grades have been seen within the prearranged ~~the~~ precincts ~~prearranged~~.

From the outcomes of this study, it was concluded ~~accomplished~~ that the presented method successfully passed ~~in~~ the precision experiment as per ICH guidelines.

Table 8. The repeatability and intermediate precision outcomes of the MTL

Conc. (µg/ml)	Intra-day precision (Repeatability)			Inter-day precision (Intermediate precision)		
	Mean area ± SD	% RSD	Inference	Mean area ± SD	% RSD	Inference
85	3077304.33 ± 60101.22	1.95	Complied	4396457.33 ± 19306.48	0.44	Complied
105	3643877.33 ± 59382.90	1.63	Complied	3284226.00 ± 44307.19	1.35	Complied
125	4396457.33 ± 19306.48	0.44	Complied	4551229.67 ± 89524.85	1.97	Complied

3.1.2.3 5 %accuracy

As per ICH guideline Q2R1, the accuracy of the method should be established at three levels with three repeated measurements at each level across the range of the method. The accuracy was estimated by using three standard concentrations of BIL and MTL at three levels across the range. The results observed for accuracy were as seen in Tables 9 and 10.

Table 9. The observations of %accuracy study for BIL

Sr. No	Conc. (µg/ml)	Mean Peak Area*	Mean Measured Conc. (µg/ml)	Accuracy (%w/w)	Inference
1	170	3251179.00 ± 54586.81	172.40	101.41	Complied
2	210	3800053.67 ± 49176.58	203.59	96.95	Complied

3	250	4557073.33 ± 47890.94	246.61	98.65	Complied

*mean of three repeated measurements.

Table 10. The observations of %accuracy study for MTL

Sr. No	Conc. (µg/ml)	Mean Peak Area*	Mean Measured Conc. (µg/ml)	% Accuracy	Inference
1	85	3053772.00 ± 60056.86	86.61	101.89	Complied
2	105	3608005 ± 32622.93	102.24	97.37	Complied
3	125	4380494 ± 59043.00	124.02	99.22	Complied

*mean of three repeated measurements.

The % accuracy of the BIL was seen in the range of 96.95-101.41 %w/w, whereas, the %accuracy ~~for~~ of MTL was observed between 97.37-101.89 %w/w. The results were observed to be in compliance with the compendia standards prescribed for BIL and MTL.

3.1.2.4 Robustness

The robustness of the present method was studied by small but purposeful variations in the method parameters, viz., detector wavelength in 'nm', the flow rate of the mobile phase in ''mL/min'', and organic concentration of the mobile phase in '%v/v'. The experimental setup of the robustness experiment was tabulated in the experimental section cited above.

The outcomes of the robustness experiment with deliberate variation in the detector wavelength were as tabulated in table 11. From the results attained, it was observed that the %assay values of the BIL and MTL were seen in the range of 98.05-99.16 %w/w and 98.38-106.25 %w/w respectively. From the results attained, it was observed that the small and deliberate variation in the detector wavelength does not affect the %assay results of BIL as well as MTL.

Further, the method was subjected to another purposeful variation in the method parameter, i.e., a change in the concentration of the organic phase of the mobile phase. The variation was set ~~as~~ at ±5%. The results acquired were as shown in table 12. From the results acquired in this study, the % assay of BIL was in the range of 98.05-101.59 %w/w. Also, the %assay for MTL was observed in the range of 98.38-99.63 %w/w. The results were seen to be in conformity with the standards.

Table 11. Results acquired for robustness experiment with variation in detector wavelength for a mixture of BIL and MTL at 200 and 100ppm respectively

λ in 'nm'	Mean peak area*		Mean measured conc. (µg/ml)		% Assay (w/w)		Inference	
	BIL	MTL	BIL	MTL	BIL	MTL	BIL	MTL
254	3668182	3471319	196.10	98.38	98.05	98.38	Complied	Complied
250	3768056	3812426	201.77	108.00	98.92	106.25	Complied	Complied
258	3776596	3642577	202.26	103.21	99.16	101.54	Complied	Complied

n = 3, *Mean peak area of three repeated measurements

Table 12. Results assimilated for robustness research with the disparity in the organic concentration of the mobile phase for a mixture of BIL and MTL at 200 and 100ppm respectively

% Org. Conc.	Mean peak area*		Mean measured conc. (µg/ml)		% Assay (w/w)		Inference	
	BIL	MTL	BIL	MTL	BIL	MTL	BIL	MTL
60	3668182	3471319	196.10	98.38	98.05	98.38	Complied	Complied
55	3781289	3515622	202.52	99.63	99.29	99.63	Complied	Complied
65	3863841	3492276	207.22	98.97	101.59	98.97	Complied	Complied

n = 3, *Mean peak area of three repeated measurements

Table 13. Results observed for robustness study with the discrepancy in flow rate (mL/min) of mobile phase for a mixture of BIL and MTL at 200 and 100ppm respectively

Flow Rate mL/min	Mean peak area*		Mean measured conc. (µg/ml)		% Assay (w/w)		Inference	
	BIL	MTL	BIL	MTL	BIL	MTL	BIL	MTL
0.6	3668182	3471319	196.10	98.38	98.05	98.38	Complied	Complied
0.45	3942344	3655394	211.68	103.57	103.77	103.57	Complied	Complied
0.75	3639347	3535492	194.46	100.19	95.33	98.38	Complied	Complied

n = 3, *Mean peak area of three repeated measurements

In addition, the method was also assessed for the effect of small but deliberate changes in the flow rate (±0.15mL/min) of the mobile phase. The measurements were carried out in triplicate and the chromatogram observed was integrated to determine the peak area and mean peak area. The percent

assay was calculated in each case of the change for BIL and MTL as illustrated in table 13. ~~The percent assay for BIL and MTL were spotted in the range of 95.33-103.77 %w/w and 98.38-103.57 %w/w respectively.~~ The percent assay for BIL and MTL was found to be in the 95.33–103.77%w/w and 98.38–103.57%w/w ranges, respectively. The method was observed to be robust even at deliberate variations in the flow rate of the mobile phase.

3.1.2.5 %Recovery / Estimation of the applicability of the method for a custom analysis of BIL and MTL as FDC in marketed pharmaceutical dosage form (tablets).

The drug content in the drug product can be studied by the percent recovery method. This also ascertains the accuracy of the method. Moreover, if the study is carried out using a marketed dosage form, it also illustrates the applicability of the method for a custom analysis of that marketed formulation(s) used for the study. In this method, the percent recovery was studied by using the marketed combined tablet dosage form of BIL and MTL.

The percent recovery experiment to determine the drug content of BIL and MTL and to ascertain the accuracy of the method was performed at three levels, viz., 80, 100, and 120% of the 100% test concentration. The percent recovery was performed by the spike method. The known amount of standard solution of a mixture of BIL and MTL was spiked into each sample solution (prepared from the tablet combined dosage form). The final test solution was injected into a given set of chromatographic conditions in triplicate and the chromatograms were recorded. The sample peak area was calculated in each case by deducting the peak area corresponding to the standard concentration spiked. The percent accuracy was then calculated by using the formula as given in the experimental section (2.2.3.5). The results observed for percent recovery of the BIL and MTL were as depicted in tables 14 ~~&~~ and 15. The representative chromatogram ~~as~~ is shown in figure 4.

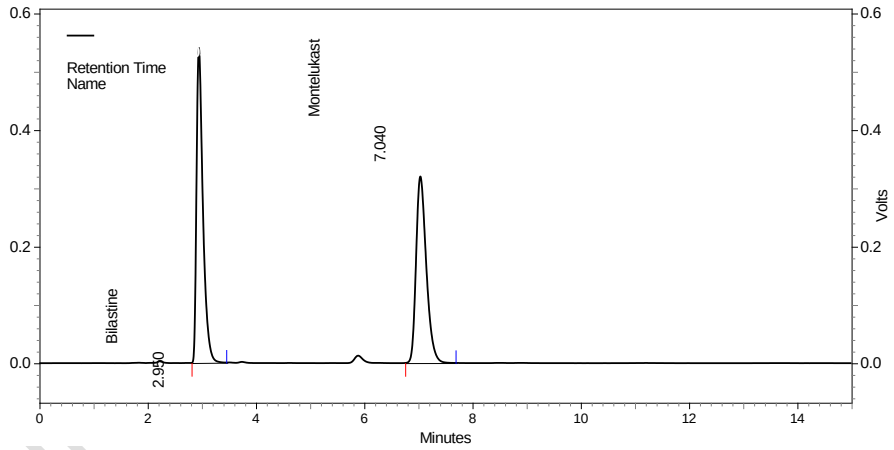

Fig. 4. Chromatogram observed in %recovery study for the test solution of BIL and MTL

As shown in table 14, the recovered amount of Bilastine was seen as, 155.81, 194.40, and 233.50 at three levels of the recovery experiment. The %recovery was observed in the range of 96.37-98.88 %w/w respectively. The recovered amount of BIL was noted in the agreement with the compendia standards.

~~Further~~ Furthermore, table 15 illustrates the results of recovery studies ~~of~~ for Montelukast. As tabulated in table 15, the recovered amount of MTL was seen as 78.06, 96.09, and 120.18 at the three respective recovery levels of the study. The percent recovery of the MTL was seen in the range of 96.11-100.06 %w/w. The results were consistent with the limits prescribed for MTL in compendia.

Table 14. Outcomes of the percent recovery experiment of BIL

% Recovery Level	Conc. of standard spiked (µg/ml)	Conc. of the sample (µg/ml)	Total mean peak area (test conc.)*	Mean peak Area of sample conc.	Amount recovered (µg/ml)	Recovery (%w/w)	Inference
80	200	160	6627484	2959302	155.81	98.88	Complied
100	200	200	7306529	3638347	194.40	97.25	Complied
120	200	240	8239472	4326387	233.50	96.37	Complied

n = 3, *three number of measurements.

Table 15. Outcomes of the percent recovery experiment of MTL

% Recovery Level	Conc. of standard spiked (µg/ml)	Conc. of the sample (µg/ml)	Total mean peak area (test conc.)	Mean peak Area of sample conc.	Amount recovered (µg/ml)	Recovery (%w/w)	Inference
80	100	80	6256492	2785173	78.06	97.70	Complied
100	100	100	6895793	3424474	96.09	96.11	Complied
120	100	120	7749790	4278471	120.18	100.06	Complied

n = 3, *three number of measurements.

Further, the retention time for Bilastine and Montelukast was found to be 2.950min.and 7.040min. respectively. The above positions of retention time for BIL and MTL in tablet dosage form were observed in conformity with that of the API mixture. ~~Hence the method was observed to be sensitive for detection of the BIL and MTL as FDC in marketed tablet dosage form in presence of allowed tablet excipients~~ As a result, the method was found to be sensitive for detecting BIL and MTL as FDC in marketed tablet dosage forms in the presence of permissible tablet excipients. Also, no supplementary peaks were seen in the chromatogram, which further confirmed the sensitivity of the method.

3.1.2.6 LOD and LOQ

In this method, the LOD and LOQ were calculated by the standard deviation of the responses obtained for all standard concentrations of Bilastine and Montelukast in the linearity investigation.

Also, the following formulae were used to calculate the LOD and LOQ of Bilastine and Montelukast.

$$\text{LOD (Bilastine)} = \frac{3.3 * 39636.49}{17595}$$

$$\text{LOQ (Bilastine)} = \frac{10 * 39636.49}{17595}$$

$$\text{LOD (Montelukast)} = \frac{3.3 * 32916.04}{35458}$$

$$\text{LOQ (Montelukast)} = \frac{10 * 32916.04}{35458}$$

The results obtained were as tabulated in table 16. As shown in table 16, the LOD and LOQ for BIL were 7.43 and 22.53µg/ml respectively. LOD and LOQ for MTL were noted as 3.06 and 9.28µg/ml respectively.

Table 16. LOD and LOQ of BIL and MTL

Standard Drug Solution	LOD (µg/ml)	LOQ (µg/ml)
Bilastine	7.43	22.53
Montelukast	3.06	9.28

3.2 Discussion

Extensive literature was explored before designing the present research work, which entitles simultaneous estimation of Bilastine and Montelukast as a mixture of API and assessment of its applicability. Peethal Pratyusha *et al* reported a UV spectroscopic method for the determination of BIL in 2020 and claimed that Beer's law was obeyed between 10-140µg/ml of BIL. The author also studied zero-order and first-order kinetics [13]. ~~Da Silva A T *et al* reported another~~ Another UV method with an experimental design for robustness in 0.1mol/liter HCl as a solvent was reported. The author claimed the precise, linear, specific, and exact [14]. Peethal Pratyusha *et al*, also reported the RP-HPLC method for the determination of BIL. The separation was achieved using formic acid and methanol in 50:50%v/v. The RT of BIL was noted to be 2.167min [15]. Pardeshi P P *et al* also reported the RP-HPLC method for analysis of BIL. Methanol and orthophosphoric acid buffer (70:30%v/v) were used as a mobile phase [16]. Firdous *et al* developed the UPLC method for estimation of BIL. The separation was achieved using buffer: methanol: acetonitrile as a mobile phase. The method has good precision and accuracy [17].

Rana *et al*, explored the RP-HPLC method for simultaneous estimation of Montelukast and Ebastine. Methanol~~:~~ , acetonitrile~~:~~ , ammonium acetate in the ratio of 80:10:10 %v/v/v ~~was~~ were used in a mobile phase to attain the separation of the components. Also, the method was successfully employed for Montelukast and Ebastine in commercially available marketed tablet dosage forms [18]. Sharma H K *et ~~al determined~~al.* determined the impurities of MTL sodium using RP-HPLC. The degradation was observed in acid and oxidative environments, whereas, it was found to be stable in other stress conditions. The separation was achieved using gradient elution [19]. Singh *et al*, estimated MTL by RP-HPLC. Acetonitrile: 1mM sodium acetate at pH 6.3 was used in the ratio of 90:10 %v/v on the C_{18} stationary phase and the detection was achieved at 285nm [20]. Gholve *et al*, further explored the quantification of the MTL using a mobile phase consisting of methanol:acetonitrile: water in ~~the~~ a ratio of 60:30:10 %v/v/v. The eluent was monitored at 344nm with RT 3.582 [21]. Murlidharan *et al*, furthered continued the work and developed HPLC and UV spectroscopic methods for estimation of MTL. One-way ANOVA was employed to analyze the results statistically. The method was successfully applied to the dosage form

[22]. Barnabas *et al* developed a novel stability-indicating method for the determination of related substances of MTL in a pharmaceutical dosage form using RP-HPLC. The separation was achieved in a gradient mode with triethylamine and acetonitrile in various combinations at pH 6.6. The method showed an excellent regression coefficient of 0.999 [23].

Besides several reports on BIL and MTL alone or in combination with other drug substances, none of the reports disclosed the simultaneous estimation of BIL and MTL in the mixture as API to explore its applicability in commercially available marketed tablet dosage forms. Hence, this research work was planned to provide a competitive method for a custom analysis of BIL and MTL for pharmaceutical tablet dosage forms.

The separation of BIL and MTL was achieved on C18 (250x4.6mm), 5µm id stationary phase by using acetonitrile: disodium hydrogen phosphate buffer in ~~the~~ a proportion of 60:40 %v/v. The elution was monitored at 254nm with a flow rate of 0.6mL/min. The analysis was carried out at 25 ºC with a run time of 15min. The optimization of the method was done by using various combinations of method parameters as shown in table 1. The final selection was as depicted above. In the above blend of mobile phases, the RT noted for BIL and MTL were 2.95 and 7.13, respectively.

The system suitability test was carried out to ensure the appropriate working of the system and it was observed that the results in terms of %RSD of mean peak area and mean RT. The %RSD was observed ~~in~~ at <0.5 for RT and <2 for mean peak area in the case of both, ~~i.e.~~ i.e., BIL and MTL. Also, other parameters were observed to be in compliance with the standards prescribed in ICH guidelines Q2R1. The ~~Linearity~~ linearity of the method was observed by injecting a series of standard concentrations of BIL MTL into the mixture. ~~The~~ A linear regression was observed for BIL and MIL with regression coefficients of 0.9971 and 0.998 respectively. The linearity of the method was observed in the range of 160-260µg/mL ~~&~~ and 80-130µg/mL with the equation of lines Y = 17595x + 217883 & Y = 35458x – 17147 for BIL and MTL correspondingly. The method was observed to be linear in the given concentration range depicted above.

The precision of the method was established using two methods, viz., repeatability and intermediate precision. The study was carried out using three standard solutions at three levels across the range. The values %RSD observed in repeatability were in the range of 0.44-1.97 and 0.44-1.95 for BIL and MTL in that order. However, the %RSD observed for intermediate precision was found in the range of 1.05-1.68 and 0.90-197. All outcomes of the precision experiment showed the %RSD values ~~in~~ within the prescribed limits (<2). Hence, the method was proved to be precise for the quantification of BIL and MTL.

The robustness of the method was carried out by purposeful variations in the method parameters, viz., detector wavelength, the organic concentration of the mobile phase, and flow rate. The results were reported in terms of percent assay of BIL and MTL and were ~~seen~~ found to be in conformity with the standards prescribed in the compendia. Further, it was ~~achieved~~ demonstrated that the small and deliberate alterations in the method parameters ~~cannot~~ could not affect the method performance for the quantification of drug substances as well as drug products. Hence, the method was robust.

The method was observed to be accurate for the estimation of BIL and MTL in the mixture at three concentration levels across the range. The percent assay results were seen in agreement for both BIL and MTL.

The %recovery of the method was established at three recovery levels of 100% test concentrations of BIL and MTL in the mixture. The % recoveries of BIL and MTL at 80, 100 and 120% levels were noted to be 98.88, 97.25, 96.37 5w/w and 97.70, 96.11, 100.06 %w/w correspondingly. All results noted were in agreement with the limits prescribed. Further, the respective peaks of the BIL and MTL were observed at the same position as those seen in the API mixture. This ~~suggested~~ suggests that the method remains unaffected by commonly used excipients in the formulation of the tablet dosage form. Also, no supplementary peaks were observed in the chromatogram. This indicated method was specific ~~for~~ to the selected drug combination of BIL and MTL. In addition, this study confirmed the applicability of the presented method for a custom analysis of BIL and MTL in pharmaceutical tablet~~s~~ dosage forms commercially available ~~in~~ on the market.

~~The low values LOD and LOQ (7.43 & 22.53 µg/ml for BIL and 3.06 & 9.28µg/ml for MTL respectively) supplementary inveterate the sensitivity of the method towards detection as well quantification of Bilastine and Montelukast.~~ The low LOD and LOQ values (7.43 & 22.53 g/ml for BIL and 3.06 & 9.28 g/ml for MTL, respectively) further impair the method's sensitivity for detection and quantification of Bilastine and Montelukast.

4. CONCLUSION

The authors have efficiently developed a simple, sensitive, specific, precise, accurate, and economic method for the quantification of Bilastine and Montelukast in the mixture as API. Further applicability of the method was assured by successfully quantifying the BIL and MTL from commercially available tablet dosage forms in the market. Also, the method was specifically designed ~~specific~~ to quantify BIL and MTL in the presence of the sample matrix. Hence, in conclusion, we have achieved all our predefined objectives ~~of~~ for this research work.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

REFERENCES

1] Kumar A, Jawla S, Yadav G. Recent analytical methods developed by RP-HPLC. Global J. Pharmacol. 2013:7(3):232-240.

2] Kumar SD, Kumar DRH. Importance of RP-HPLC in analytical method development: A Review. IJPSR. 2012:3(12):4626-4633.

3] Chensheng L, Sitaram B, Marie PT Melanson J, Blomgren A, Rundlöf T, Kilpatrick E. et al. Survey of peptide quantification methods and comparison of their reproducibility. Journal of pharmaceutical and biomedical analysis. 2019:166:105-112.

4] Chunwei M, Yan R, Jiarui Y, Zhe R, Huanming Y, Siqui L. Improved peptide retention time prediction in liquid chromatography through deep learning. 2018:90(18):10881-888.

5] Branko N, Belma I, Saira M, Miroslav S. High-performance liquid chromatography in pharmaceutical analysis. Bosn J Basic Med Sci. 2004:4(2):5-9

6] Baiocchi C, Marchetto A, Saini G, Bertolo P, Pettiti G. Reverse-phase HPLC separation of complex mixtures of trace metals as dibenzyl dithiocarbamate chelates. 1988:35(9):685-691.

7] Ridolo E, Montagni M, Bonzano L, Incorvaia C, Canonica GW. Bilastine: New insight into antihistamine treatment. 2015:13(1):1-6.

8] Katherine A, Lyseng W, Carter NJ, Bilastine: a guide to its use in the treatment of symptomatic allergic rhinoconjunctivitis and urticarial. 2012:28:1-5.

9] Krause K, Spohr A, Zuberbier T, Church MK, Maurer M. up dosing with bilastine results in improved effectiveness in cold contact urticaria. Allergy. 2013:68(7):921-928.

10] Ramires R, Caiaffa MF, Tursi A, Haeggstrom JZ, Macchia L. Novel inhibitory effect on 5-lipoxygenase activity by the anti-asthma drug montelukast. Biochem Biophys Res Commun. 2004: 324(2):815-21.

11] Paggiaro P, Bacci E. Montelukast in Asthma: A Review of its Efficacy and Place in Therapy. Ther Adv Chronic Dis. 2011:2(1): 47-58.

12] Luo H, Han H, Liu X, Liu Q. Efficacy and safety of montelukast sodium combined with fluticasone in the treatment of adult bronchial asthma: A protocol for systematic review and meta-analysis, medicine. 2020:52:1-5.

13] Peethala P, Sundararajan R. UV spectrophotometric method for determination of Bilastine in bulk and pharmaceutical formulation. 2020:13(2):933-938 doi:10.5958/0974-360X.2020.00176.6.

14] da Silva AT, Gabriela RB, Marques ID, Bajerski L, Malesuik MD, Paim CS. UV Spectrophotometric Method for the quantitative determination of Bilastine using experimental design for robustness. Drug Anal Res, 2017: 01(2):38-43.

15] Peethala P, Sundararajan R, Bhanu P, Mukthinuthalapati MA. New stability indicating RP-HPLC method for determination of Bilastine in bulk and pharmaceutical formulation. Research J. Pharm. and Tech 2020:13(6):2849-2853 DOI: 10.5958/0974-360X.2020.00507.7.

16] Pardeshi PP, Gaware VM, Dhamak KB, Development and Validation of RP-HPLC Method for the Estimation of Bilastine from bulk and Formulation. Asian J. Pharm. Ana. 2020; 10(2):109-111. DOI: 10.5958/2231-5675.2020.00019.8.

17] Firdous S, Rizwan SH. Analytical method development and validation for the estimation of bilastine in bulk and pharmaceutical dosage form by UPLC. 2020:6(10):138-143.

18] Rana NS, Rajesh KS, Patel NN, Patel PR, Limbachiya U, Pasha TY. Development and Validation of RP- HPLC Method for the Simultaneous Estimation of Montelukast Sodium and Ebastine in Tablet Dosage Form. Indian J Pharm Sci.: 2013:75(5):599-602 PMID: 24403662.

19] Sharma HK, Rashmitha N, Raj TJS, Srinivas CH, Srinivas N, Ray UK, Mukkanti K. A Validated RP-HPLC Method for the Determination of Impurities in Montelukast Sodium. E-Journal of Chemistry: 2010:7(2): 555-563.

20] Singh RM, Saini PK, Mathur SC, Singh GN, Lal B. Development and Validation of an RP-HPLC Method for Estimation of Montelukast Sodium in Bulk and Tablet Dosage Form. Indian J Pharm Sci.: 2010:72(2):235-237. DOI: 10.4103/0250-474X.65023. PMID: 20838530.

21] Gholve S, Thonte S, Bhusnure O. RP-HPLC method development and validation of montelukast sodium in bulk drug and dosage form. Int J Pharm Bio Sci.:2015: 6(2): 354 – 360.

22] Muralidharan S, Qi LJ, Yi LT, Kaur N, Parasuraman S, Kumar J, Venugopal V, Raj PV. Newly Developed and Validated Method of Montelukast Sodium Estimation in Tablet Dosage Form by Ultraviolet Spectroscopy and Reverse Phase-High Performance Liquid Chromatography. PTB Reports:2016:2(2): 27-30.

23] Barnabas KS, Suvaitha SP, Dhinagaran G, Venkatachalam K. A Novel Stability-Indicating Method for Determination of Related Substances of Montelukast Sodium in a Pharmaceutical Dosage Form Using RP-HPLC. Chromatographia.: 2021:84: 645–662.