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A New Simple RP-HPLC Method Development, Validation and Stability Studies for the Simultaneous Estimation of Montelukast and Ebastine in Pure Form and Combined Tablet Formulation

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Abstract
Objective: The objective of the present research work was to develop an innovative, simple, and economic method for estimation of Montelukast and Ebastine in bulk and dosage form by RP-HPLC. **Methods:** The chromatographic conditions were performed on Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Acetonitrile: Phosphate buffer (pH – 3.0) 1.0 ml/min, with Injection Volume 20µl, at detection wavelength 255 nm and run time at 14.0 min. **Results:** The analytical method is valid for estimation of Montelukast and Ebastine over a range of 0-16 µg/ml, 0-35 µg/ml. The results of system suitability test, linearity, precision and accuracy, robustness, specificity, LOD and LOQ and stabilities presented in this report are within the acceptance range. **Conclusion:** A specific, sensitive, economic method estimation of Montelukast and Ebastine has been developed based on ICH Guidelines with bulk and dosage forms.

Keywords
Montelukast and Ebastine, HPLC, Method Development, ICH, Validation, Accuracy, Precision.

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1. INTRODUCTION
Montelukast Sodium (MNK) chemically, (S, E) −2-(1-((1-(3-(2-(7-chloroquinolin-2-yl) vinyl) phenyl) 3-(2 - (2 - hydroxypropan - 2yl) phenyl) propylthio) methyl) cyclopropyl) acetic acid [1,2] is a cysteinyl leukotriene receptor antagonist used for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies [3-5].

Literature survey reveals that assay of MNK in bulk and tablet dosage form is official in Indian Pharmacopoeia 2010[6].

IUPAC Name: 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.

Ebastine (EBA), chemically, 4-(4-benzhydryloxy-1-piperidyl)-1-(4-tert-butylphenyl) butan-1-one is a no sedating H1 antihistamine. Assay of ebastine in bulk form is official in British Pharmacopoeia [7].

Literature survey reveals that analytical methods, including UV spectrophotometer, and HPLC methods, are available for the determination of montelukast in pharmaceutical dosage forms. The analytical methods reported for estimation of MNK and EBA alone and with other drug combination are UV spectrophotometry [8 - 11], HPLC [12 - 14], HPLC/PDA [15], LC - MS [16] and HPTLC [17 - 19] methods. The combination of MNK and EBA has recently been introduced into the market. However, so far, no method was reported for the simultaneous estimation of MNK and EBA, in combination. The proposed method is rapid, simple, accurate and reproducible and can be successfully employed in the routine analysis of both these drugs simultaneously, in tablet dosage form. The proposed method is optimized and validated as per the ICH guidelines [20]. In the present work, a successful attempt has been made to estimate both these drugs simultaneously using RP-HPLC method. This study attempts to describe a rapid and sensitive HPLC method with UV detection, useful for routine quality control of MNK and EBA in pharmaceutical formulation.

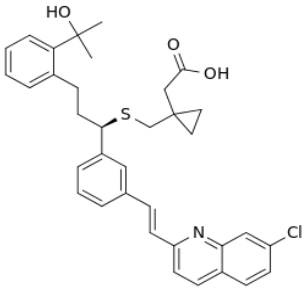


Fig no.1-Structure of Montelukast

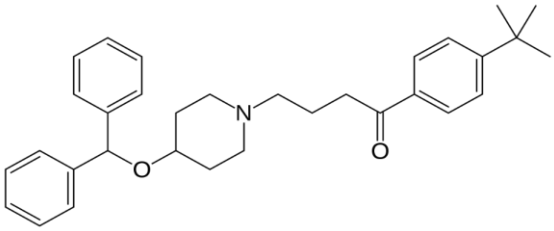


Fig no.2- Structure of Ebastine

2. Experimental

2.1 Materials and Methods:

Pharmaceutical grade working standard Montelukast and Ebastine were obtained from Syncorp Pvt. Laboratories, Hyderabad, India. All chemicals and reagents were HPLC grade and were purchased from S D Fine-Chem Limited & Loba Chemie Pvt Ltd, Mumbai, India.

2.2 Instrumentation:

The analysis was performed using HPLC (Waters-717 series) with PDA detector and data handling system EMPOWER2 software, UV-Visible double beam spectrophotometer (ELICO SL-159), analytical balance 0.1mg Sensitivity (SHIMADZU), pH meter (Labindia), ultra sonicator. The column used is Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d. (as Stationary phase) with the flow rate 1.0ml/min (isocratic).

2.3 Sample and Standard Preparation for the Analysis

Accurately weighed 10mg of Montelukast and 10mg of Ebastine working standard were transferred intoa10mL and 10ml of clean dry volumetric flasks. About 7mL and 70ml of Diluents are added and sonicated to dissolve it completely and made volume up to the mark with the same solvent. (Stock solution) Further 3ml of Montelukast and 0.03ml of Ebastine the above stock solution was pipette into a 10ml volumetric flask and diluted up to the mark with diluents.

2.4 Selection of wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent. (After optimization of all conditions) for UV analysis. It is scanned in the UV spectrum in the range of 200 to 400nm. This has been performed to know the maxima of Montelukast and Ebastine, so that the same wave number can be utilized in HPLC UV detector for estimating the Montelukast and

Ebastine. While scanning the Montelukast and Ebastine solution we observed the maxima at 255 nm.

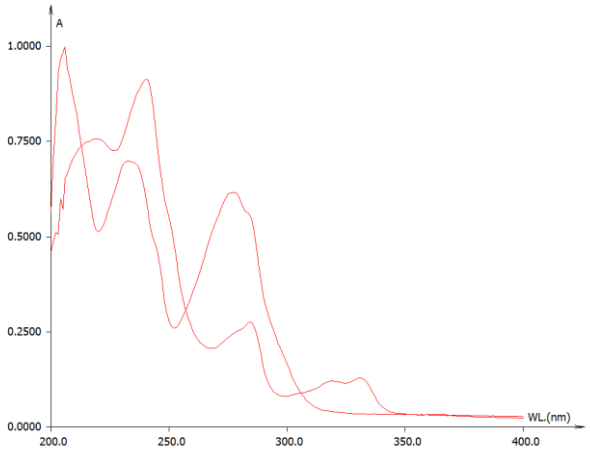


Fig-2: UV Spectrum for Montelukast and Ebastine

2.5 Method Development

2.5.1. Preparation of Phosphate buffer(PH:3.0):

Mix 0.7ml of Phosphoric acid with 100ml of water. Dilute to 900ml with the same solvent. Adjust to pH 3.0 with strong sodium hydroxide solution and dilute to 1000ml with water.

2.5.2 Preparation of Mobile Phase:

The mobile phase was prepared with the combination of Acetonitrile and phosphate buffer (3.0) at the volume of 1000ml. 750ml of Acetonitrile and 250 of Phosphate buffer (3.0) were mixed well and degassed in ultrasonic water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

2.5.3 Summary of Optimized Chromatographic Conditions:

The Optimum Chromatographic conditions obtained from experiments can be summarized as below:

Table-1: Summary of Optimised Chromatographic Conditions

Mobile phase	Acetonitrile : Phosphate buffer(3.0)
Column	Phenomenex Luna C_{18}, 100A, 5µm, 250mmx4.6mm i.d.
Flow rate	1.0 ml/ min.
Wavelength	255nm
Sampling System	Automatic
Temp. of Auto sampler	Ambient
Volume of injection	20µl
Run time	14 min.
Mode of Separation	Isocratic

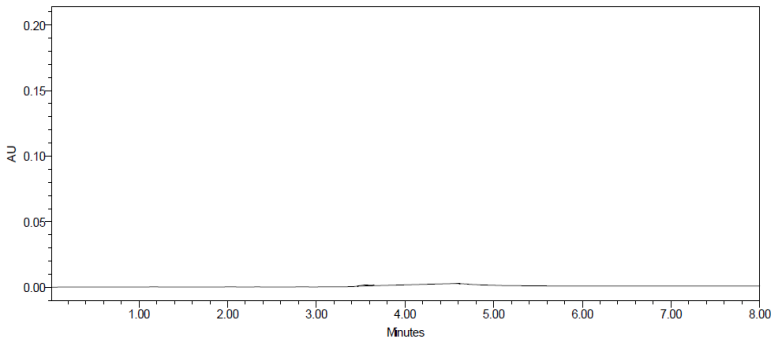
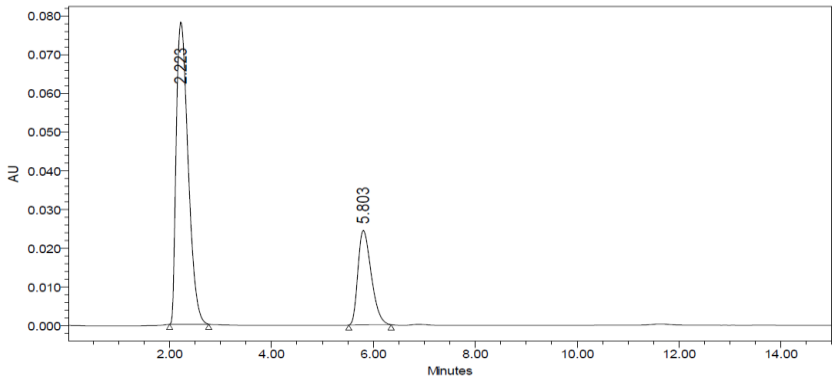


Fig-3: Chromatogram for Blank Preparation



Fig–4: Chromatogram of Montelukast and Ebastine in Optimized Condition

2.6 Method validation:

2.6.1 Linearity & Range:

Calibration standards at five levels were prepared by appropriately mixed and further diluted standard stock solutions in the concentration ranges from 0-16µg/ml for Montelukast and concentration ranging from 0-35µg/ml for Ebastine. Samples in triple injections were made for each prepared concentration. Peak areas were plotted against the corresponding concentration to obtain the linearity graphs. Chromatograms of each solution were recorded.

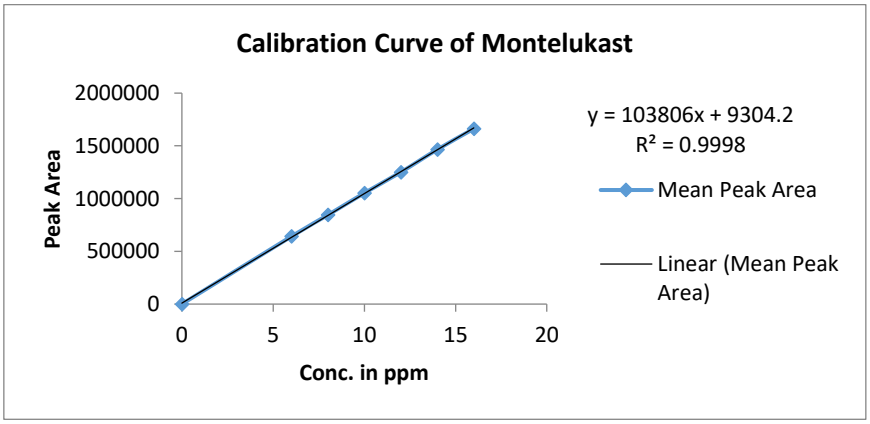


Fig-8: Standard curve for Montelukast

Table-2: Linearity Results for Montelukast

S. No.	Conc. (µg/ml)	Mean Peak Area
1	0	0
2	6	641233
3	8	844610
4	10	1052647
5	12	1250435
6	14	1465354
7	16	1662043

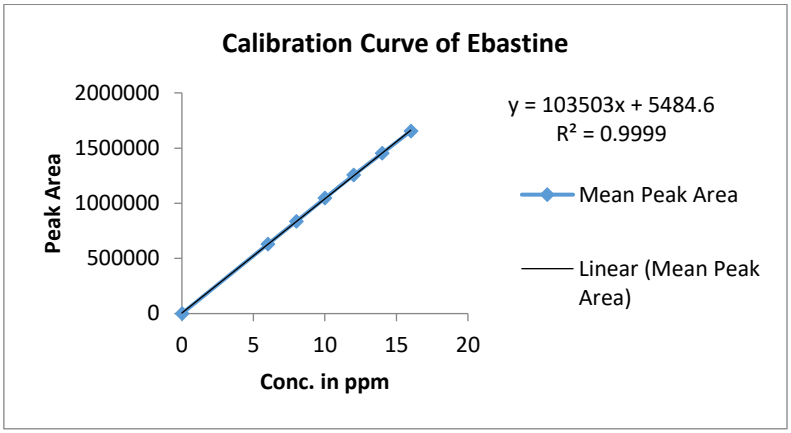


Fig-9: Standard curve for Ebastine

Table-3: Linearity Results for Ebastine

Sr. No.	Conc. (µg/ml)	Mean Peak Area
1	0	0
2	10	628423
3	15	835412
4	20	1045742
5	25	1254033
6	30	1452471
7	35	1653504

2.6.2. Accuracy:

Recovery study*:* Montelukast

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of MONTELUKAST were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y =10380x + 9304.

Recovery study: Ebastine

To determine the accuracy of the proposed method, recovery studies were carried out by adding different amounts (80%, 100%, and 120%) of pure drug of EBASTINE were taken and 3 replications of each has been injected to HPLC system. From that percentage recovery values were calculated from the linearity equation y = 10350x + 5484.

Table-4: Accuracy Readings for Montelukast

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.105	93435	101.312	Mean= 100.0163%
S_2 : 80 %	8	7.898	91287	98.725	S.D. = 1.293505
S_3 : 80 %	8	8.001	92356	100.012	% R.S.D.= 1.293294
S_4 : 100 %	10	10.195	115135	101.95	Mean= 101.4033%
S_5 : 100 %	10	10.152	114687	101.52	S.D. = 0.613379
S_6 : 100 %	10	10.074	113879	100.74	% R.S.D.= 0.60489
S_7 : 120 %	12	12.171	135647	101.425	Mean= 100.6053%
S_8 : 120 %	12	12.044	134324	100.366	S.D. = 0.730041
S_9 : 120 %	12	12.003	133897	100.025	% R.S.D. = 0.725649

Table no.5: Accuracy Results for Ebastine

Sample ID	Concentration (µg/ml)			%Recovery of Pure drug	Statistical Analysis
	Conc. Found	Conc. Recovered	Peak Area		
S_1 : 80 %	8	8.100	89325	101.25	Mean= 100.1207%
S_2 : 80 %	8	8.027	88569	100.337	S.D. = 1.251602
S_3 : 80 %	8	7.902	87279	98.775	% R.S.D.= 1.250093
S_4 : 100 %	10	10.122	110254	101.22	Mean= 101.44%
S_5 : 100 %	10	10.128	110312	101.28	S.D. = 0.330454% R.S.D.= 0.325763
S_6 : 100 %	10	10.182	110874	101.82	
S_7 : 120 %	12	12.147	131215	101.225	Mean= 101.444%
S_8 : 120 %	12	12.161	131356	101.341	S.D. = 0.284828
S_9 : 120 %	12	12.212	131879	101.766	% R.S.D. = 0.280774

2.6.3. Precision:

2.6.3.1. Repeatability

The precision of each method was ascertained separately from the peak areas & retention times obtained by actual determination of six replicates of a fixed amount of drug Montelukast and Ebastine (API). The percent relative standard deviation was calculated for Montelukast and Ebastine are presented in the Table-4.

Table-6: Data showing repeatability analysis for Montelukast and Ebastine

HPLC Injection Replicates	AUC for Montelukast	AUC for Ebastine
Replicate – 1	1013546	1035681
Replicate – 2	1025824	1065897
Replicate – 3	1012351	1078953
Replicate – 4	1036584	1058748
Replicate – 5	1015419	1078754
Replicate – 6	1008572	1065871
Average	1018716	1063984
Standard Deviation	10495.73	15986.99
% RSD	1.03029	1.50256

2.6.3.2. Intermediate precision:

The intra & inter day variation of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Montelukast and Ebastine revealed that the proposed method is precise.

Table-7: Data for Montelukast Analysis

Conc. Of Montelukast (API) (µg/ml)	Observed Conc. Of Montelukast (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.03	0.25	9.95	0.21
10	10.49	0.36	10.02	0.32
12	11.14	0.14	12.30	0.19

Table-8: Data for Ebastine analysis

Conc. Of Ebastine (API) (µg/ml)	Observed Conc. of Ebastine (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=3)	% RSD	Mean (n=3)	% RSD
8	8.05	1.02	9.59	0.98
10	9.94	0.74	10.09	0.56
12	11.97	0.35	12.006	0.32

2.6.4. Method Robustness:

Influence of little changes in optimized chromatographic conditions like changes in flow rate (± 0.1ml/min), mobile phase ratio (±2%), Wavelength of detection (±2nm) and Acetonitrile content in mobile phase (±2%) studied to measure the robustness of the method are also in favour of (Table-36, % RSD < 2%) the developed RP-HPLC method for the analysis of Montelukast and Ebastine (API).

Table-9: Result of Method Robustness Test for Montelukast

Change in parameter	% RSD
Less Flow (0.8 ml/min)	1.06
More Flow (1.2 ml/min)	0.69
More Organic	0.45
Less Organic	0.56
Wavelength of Detection (238 nm)	0.28
Wavelength of detection (242 nm)	0.14

Table no-10: Result of Method Robustness Test for Allopurinol

Change in parameter	% RSD
Flow (0.8 ml/min)	0.03
Flow (1.2 ml/min)	0.08
More Organic	0.19
Less Organic	0.73
Wavelength of Detection (271 nm)	0.82
Wavelength of detection (267 nm)	0.46

2.6.5. LOD & LOQ:

The detection limit (LOD) and quantitation limit (LOQ) may be expressed as:

L.O.D. = 3.3(SD/S).

L.O.Q. = 10(SD/S)

Where, SD = Standard deviation of the response

S = Slope of the calibration curve

2.6.6. System Suitability Parameter

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. Following system suitability test parameters were established. The data are shown in Table-12.

Table-11: Dataof System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs> 2	3.56
2	Asymmetry	T ≤ 2	Montelukast = 0.17 Ebastine = 1.01
3	Theoretical plate	N > 2000	Montelukast = 3698 Ebastine = 4926

2.6.7 Estimation of Montelukast and Ebastine in Tablet Dosage Form

Twenty tablets were taken and the I.P. method was followed to determine the average weight. Finally, the weighed tablets are powdered and triturated well by using mortar and pestle. A quantity of powder which is equivalent to the 100mg of drugs were transferred to a clean and dry 100ml of volumetric flask and add 70 ml of mobile phase and the resulted solution was sonicated for 15 minutes by using ultra sonicator, Then the final volume was make up to the mark with the mobile phase. The final solution was filtered through a selected membrane filter (0.45 µm) and in order to sonicated to degas the mobile phase (Solvent system). From this above stock solution (1 ml) was transferred to five different 10 ml volumetric flasks and volume was made up to 10 ml with same solvent system (Mobile phase).

A duplicate injection (Blank Solution) of the standard solution also injected into the HPLC system and the chromatograms and peak areas were recorded and calculated. The obtained data are shown in the chapter results and discussion.

Table-12: Assay of marketed formulation

Brand name of Montelukast and Ebastine	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Ebafix-M (Strivos Pharma)	10/10	9.96 (±0.438) / 9.92 (± 0.422)	99.6(±0.543) / 99.2(± 0.462)

2.6.8 Stability studies:

The API (Montelukast and Ebastine) was subjected to stress conditions in various ways to observe the rate and extent of degradation that is likely to occur in the course of storage and/or after administration to body. The various degradation pathways studied are acid hydrolysis, basic hydrolysis, thermal degradation, photolytic degradation and oxidative degradation.

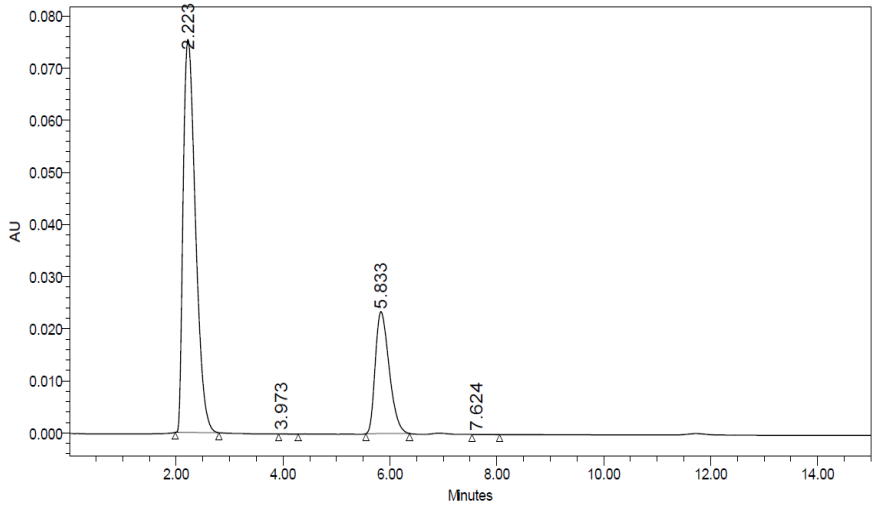


Fig-5: Chromatogram for Acid Degradation

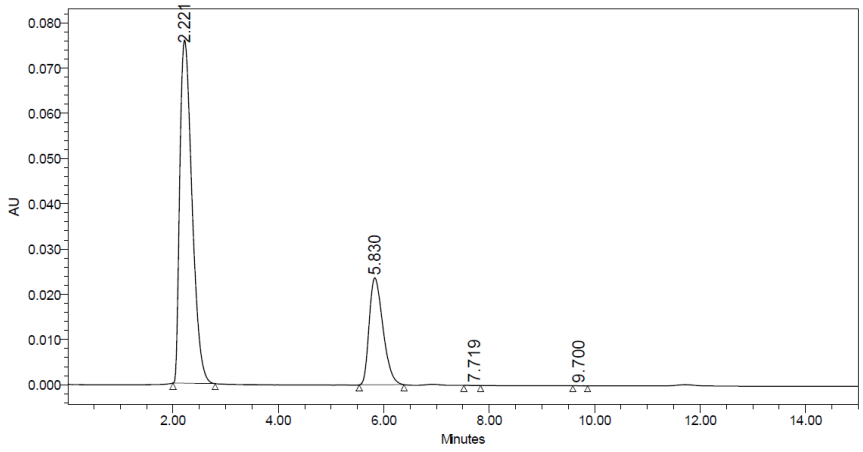


Fig-6: Chromatogram for Basic Degradation

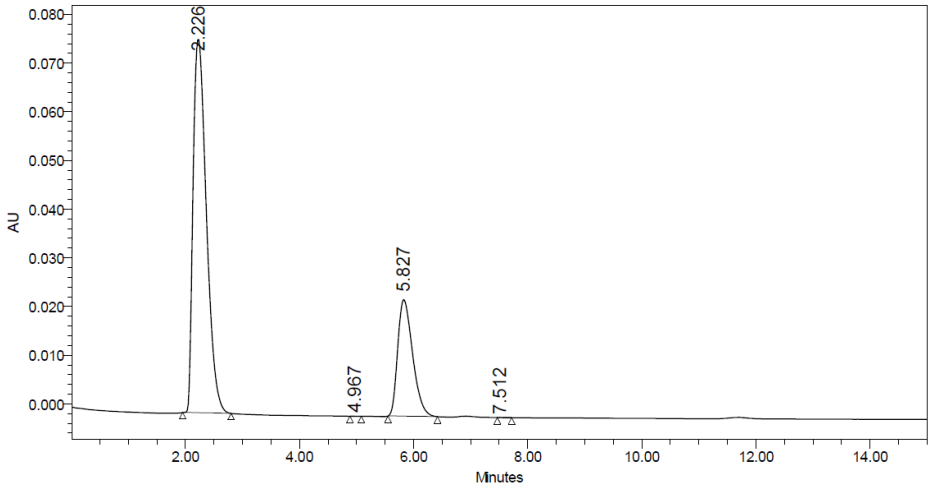


Fig-8: Chromatogram for Thermal Degradation

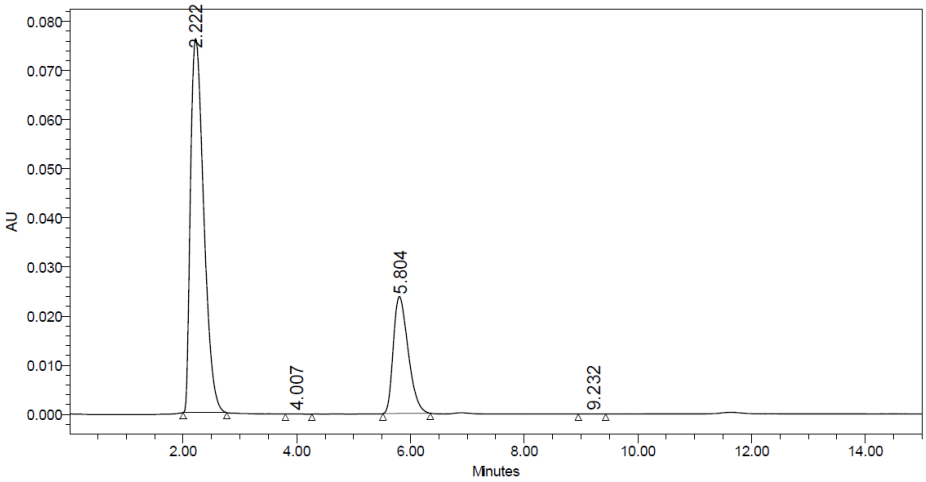


Fig-9: Chromatogram for Photolytic Degradation

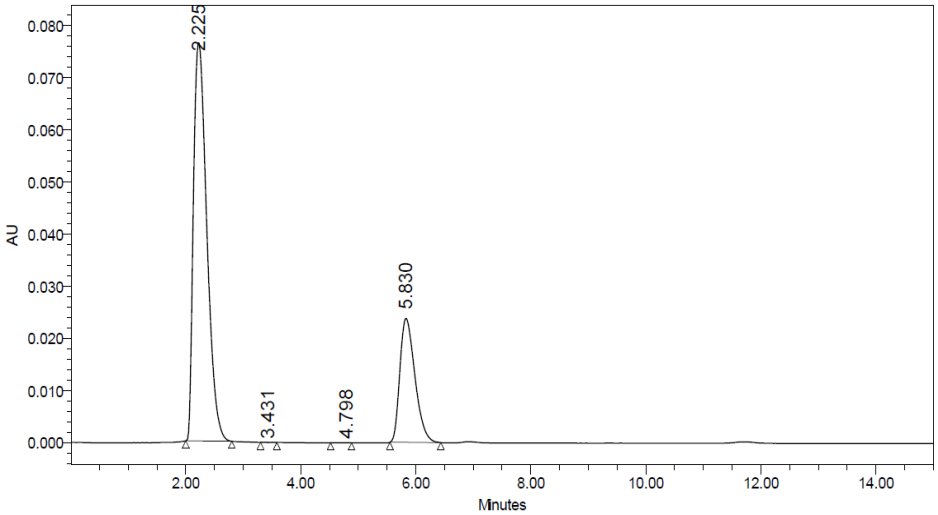


Fig-10: Chromatogram for Oxidation with 3% H_2O_2 Degradation

Table-13: Results of Stress studies of Montelukast API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	95.71	4.86	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.63	2.93	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	96.42	3.72	100.00
UV (254nm)	24Hrs.	95.52	4.63	100.00
3% Hydrogen peroxide	24Hrs.	96.05	3.48	100.00

Table-14: Results of Force Degradation Studies of Ebastine API.

Stress condition	Time (hours)	Assay of active substance	Assay of degraded products	Mass Balance (%)
Acid Hydrolysis (0.1N HCl)	24Hrs.	94.35	5.86	100.00
Basic Hydrolysis (0.IN NaOH)	24Hrs.	97.42	2.72	100.00
Thermal Degradation (60 ^{0}C)	24Hrs.	98.43	1.63	100.00
UV (254nm)	24Hrs.	93.27	6.93	100.00
3% Hydrogen peroxide	24Hrs.	96.39	3.10	100.00

3. RESULTS:

The optimized chromatographic conditions were Phenomenex Luna C18, 100A, 5µm, 250mmx4.6mm i.d.as stationary phase and mobile phase was prepared with a mixture of Acetonitrile and Phosphate buffer (3.0)) flow 1.0 ml/min, with Injection Volume 20µl, at detection wavelength 255 nm and run time at 14.0 min. In these chromatographic conditions the peak was pure, sharp, symmetric and found a greater number of theoretical plates.

The results obtained in method validation were

Linearity & Range: Linearity range was found to be 0-16 µg/ml for Montelukast. The correlation coefficient was found to be 0.999, the slope was found to be 10380 and intercept was found to be 9304 for Montelukast.

Linearity range was found to be 0-35 µg/ml for Ebastine. The correlation coefficient was found to be 0.999, the slope was found to be 10350 and intercept was found to be 5484 for Ebastine.

Accuracy:

Montelukast: From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.0163%, 101.4033% and 100.60531% which is within the range of 98-102% and %RSD is within the range <2 i.e. 1.293294%, 0.60489% and 0.725649% respectively.

Ebastine: From the Accuracy Method, we observed that the mean %Recovery of the drug are 100.1207%, 101.44% and 101.44% which is within the range of 98-102% and %RSD is within the range <2 i.e. 1.250093%, 0.325763% and 0.280774 % respectively.

Repeatability: The repeatability study which was conducted on the solution having the concentration of about 10 µg/ml for Montelukast and 10 µg/ml for Ebastine (n =6) showed a RSD of 1.03029% for Montelukast and 1.50256% for Ebastine. It was concluded that the analytical technique showed good repeatability.

LOD & LOQ: The LOD was found to be 0.607 µg/ml and 1.821 µg/ml and LOQ was found to be 0.451

µg/ml and 1.353 µg/ml for Montelukast and Ebastine respectively which represents that sensitivity of the method is high.

Assay: The assay of Ebafix-M Tablets containing 10mg of Montelukast &10mg of Ebastine was found to be 99.6% and 99.20%respectively.

Degradation studies: The results of the forced degradation studies indicated the specificity of the developed method that has been developed. Montelukast and Ebastine were stable only in oxidative stress conditions and photolytic stress conditions. The results of stability studies are given in the following Table-13 and Table -14.

4. DISCUSSION

To develop a precise, linear, specific RP-HPLC method for analysis of Montelukast and Ebastine, different chromatographic conditions were applied & the results observed were compared with the methods available in literatures.

Dr. Hepcy Kalarani D* et al., [21] Development and validation of reverse phase HPLC method for the simultaneous estimation of montelukast and ebastine in its tablet dosage form. The reverse phase HPLC includes stationary phase is non-polar hydrophobic packing with octyl (or) octa decyl functional group bonded to silica gel and mobile phase is polar solvent. Montelukast is used regularly to prevent the wheezing and anti-asthma. Ebastine is an anti-histamine.**N. S. Rana* et al., [22]** A rapid and sensitive RP‑HPLC method with UV detection (244 nm) for routine analysis of montelukast sodium and ebastine in a pharmaceutical formulation (Ebast‑M) was developed. Chromatography was performed with mobile phase containing a mixture of methanol:acetonitrile:ammonium acetate (80:10:10, % v/v/v), pH of mobile phase was adjusted 5.5 using glacial acetic acid and flow rate was 1.2 ml/min. The method was validated for linearity, accuracy, robustness and intermediate precision. The linearity was established over the concentration range of 0.01–0.06 mg/ml for both drugs. The correlation coefficients (r2) for ebastine and montelukast were 0.9989 and 0.9955, respectively. **Anand Jigna* et al., [23]** A specific and accurate reverse phase high performance liquid chromatographic method was developed for the simultaneous determination Ebastine (EBA) and Montelukast Sodium (MONTE) in Combined dosage forms. The analysis was carried out using Phenomenex C18, column (250 mm × 4.6 mm id, 5 µm particle size) with Mobile phase consisting of Methanol: Phosphate buffer (65:35 v/v) pH 5.0+0.05 was pumped at a flow rate was 1.0 ml/ min and Quantification was achieved with photodiode array (PDA) detection at 261 nm over the concentration range of 10 - 50 µg/mL for ebastine & Montelukast Sodium with mean recovery of 100.32 ± 0.85 % and 100.15 ± 0.94 % for Ebastine and Montelukast Sodium, respectively. **Jyoti J. Savsani* et al., [24]** Simultaneous Equation Method for simultaneous estimation of Ebastine and Montelukast sodium in combined tablet dosage form has been developed. The UV spectrophotometric method is the simultaneous equation method which involves the formation of absorbance equation at 253nm maximum absorption of Ebastine and at 344nm the maximum absorption of Montelukast Sodium. The linearity ranges for Ebastine and montelukast sodium were 5-45µg/ml and 5-45 µg/ml respectively. The accuracy of the method was assessed by recovery studies was found to be and 100.43 ± 0.1893 and 100.22 ± 0.3215 for simultaneous equation method for ebastine and montelukast sodium respectively.

The result shows the developed method is yet another suitable method for assay which can help in the analysis of in formulations.

5. CONCLUSION

A sensitive & selective stability indicting RP-HPLC method has been developed & validated for the analysis of Montelukast and Ebastine API. Based on peak purity results, obtained from the analysis of samples using described method, it can be concluded that the absence of co-eluting peak along with the main peak of Montelukast and Ebastine indicated that the developed method is specific for the estimation of Montelukast and Ebastine. Further the proposed RP-HPLC method has excellent sensitivity, precision and reproducibility.

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