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Application of Design of Experiment in Design, Development and Optimization of Stability Indicating RP-HPLC Method for Simultaneous Determination of Montelukast Sodium and Rupatadine Fumarate in Bulk and Formulation

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Authors' contributions

This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.

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ABSTRACT

Design of Experiment assisted stability indicating RP-HPLC wasdesigned, developed and optimized using response surface methodology for simultaneous determination of Montelukast sodium and Rupatadine fumarate. Separation was achieved using Acetonitrile: Phosphate buffer (75:25) v/v with pH adjusted to 4.0, flow rate of 1 ml/min with UV detection at 246 nm on RP-C18 column. Stress degradation studies were performed as per scientific guidelines. Method was validated in accordance with regulatory requirements. Results obtained in validation were found to be within specified limit. Montelukast was eluted at 3.99 min and Rupatadine was eluted at 13.25 min respectively. All stress degradation products are very well resolved from drug peak which indicate suitability indicating nature of the developed method. Design of Experiment technique can help in fast and economical optimization of mobile phase which in turn will save time for method development. The developed method is, accurate, sensitive which can be utilized as stability indicating method for identification of degradation products in routine analysis of the drug.

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Keywords: Response surface; montelukast; rupatadine; stability indicating; stress studies.

1. INTRODUCTION

Rupatadine Fumarate is an Anti-allergic, anti-histaminic molecule chemically it is 8-Chloro-6,11-dihydro-11-[1-[(5-methyl-3-pyridinyl)methyl]-4-piperidinylidene]-5*H*benzo[5,6]cyclohepta[1,2-b]pyridine fumarate. Montelukast is an leukotriene receptor antagonist and chemically it is R-(E)-1-[1-[3-[2-(7-chloro-2-quinolinyl) ethenylphenyl]-3-[2(1-hydroxy-1-methylethyl) phenylpropyl thiomethyl cyclopropane acetic acid, and is available in monosodium salt form.Both drugs are used in combination for the treatment of asthma and as anti-allergic [1-2].

Literature survey reveals that various analytical methods including, UV-Spectrophotometric [3-5], RP-HPLC [6-14], HPTLC [15] EI-GC-MS [16], LC-MS-MS [17] and MEKC [18] are available for determination of Rupatadine alone and in combination with other molecules, Montelukast sodium was determined with UV-Spectrophotometric [19-22],Stability indicating RP-HPLC [23-25], HPLC [26-34], UPLC [35] and Voltametric [36] methods either alone or in combination with other drug molecules.Simultaneous estimation of Montelukast and Rupatadine was also reported by UV-Spectroscopic, and chromatographic method, one stability indicating RP-HPLC method was also reported for simultaneous estimation of these drugs [13,36,31]. But there was no method reported which was developed and optimized with help of Design of Experiment and Response surface methodology.

Present work focusses on use of Design of Experiment technique with response surface methodology as a systematic tool fordesign, development, optimization and validation of stability indicating RP-HPLC method for simultaneous determination of Rupatadine fumarate and Montelukast Sodium.

2. MATERIALS AND METHODS

Gift samples of Montelukast Sodium and Rupatadine Fumarate were supplied by local pharmaceutical industries. Acetonitrile and Methanol (HPLC Grade were procured from Merk Specialties Pvt. Ltd., Mumbai, and Thomas Baker (chemicals) Pvt. Ltd., Mumbairespectively. Other AR grade chemicals were procured form Research Lab Fine Chem. Industries, Mumbai.

2.1 Instrument and Software

Carry-500 Double beam UV-Visible spectrophotometer (Varian) with CARRY software, HPLC Systemhaving 515-pumps with column oven, PDA detectors Auto sampler (Waters) with Empower 2.0 software and pH Meter (Equiptronics) were used for method development. Along with this Design Expert 7.0 (Trial Version)was used for designing and optimizing the method parameters. Microsoft excel was used for statistical analysis.

2.2 Experimental Section

As a first step of method development solubility of both drugs was tasted in different solvents to obtain a common solvent which can be used for simultaneous estimation of both drugs in a mixture.

2.3 Preparation of Standard Stock Solution

Standard Stock Solutions of Montelukast sodium and Rupatadine fumarate were prepared by transferring accurately weighed 100 mg of drugs to separate 100 ml volumetric flasks, enough methanol was added to the flasks and flasks were swirled for 5 min then final volume was made up to the mark with help of methanol. The concentration of resulting solution was found to be 1 mg/ml. These stock solutions were further diluted to get desired concentration for experimental work.

2.4 Preparation of Sample Stock Solution

Formulation sample (Smarti-M, German Remedies) was prepared by accurately weighing twenty tablets containing both drugs in defined ratio (Rupatadine10 mg+ Montelukast sodium 10mg), these tablets were powdered and amount of powder equivalent to 10 mg of Montelukast and 10 mg Rupatadine was transferred to a volumetric flask. The flask was ultra-sonicated for ten minutes after adding enough methanol to the flask. Final volume was made up to the mark and then solution was filtered through Whatman filter paper no 41. This process will separate the excipients from the API (Active pharmaceutical ingredient). Resulting solution was further used for experimental runs after diluting with suitable solvent.

2.5 Optimization of Chromatographic Conditions with Design of Experiment [37-39]

Earlier reported HPLC method were developed with traditional method development technique that involve changing variable based on the previous knowledge or drug properties. To minimize this trial of mobile phase optimization for method development in HPLC. New technique of systematic design of experiment was implemented for optimization of the mobile phase. This approach is known as design of Experiment approach in which important variable which causes change in retention behavior of the drug candidate were used to develop an optimized mobile phase.

2.6 Design of Experiment

First step in DoE experiment was identification of important variable that causes changes in the retention behavior of the drug. From the earlier trial runs and literature review it was found that percentage of organic phase,(%Acetonitrile), pH of the buffer solution, temperature are the important variables that should be considered in the design of experiments. Upper and lower limits of these factors were determined by trial runs on the HPLC system.

The next step in the analysis was designing a central composite design (CCD) model which was used for estimating possible combinations of the three factors, a set of 20 experiments with different values for 3 variables was obtained in CCD model this model was used to evaluate the complete set of main effects and interactions. The objective of designing these experiments was to separate the degradation peaks from main drug peak with sufficient resolution. Flow rate, Percentage of organic phase (ACN) and pH of buffer are the three variables likely to have a significant impact on the separation behavior of drug. Resolution Value of Asymmetry factor and retention time are chosen as response variables. In the design of experiment, low- and high-level values of the variables were chosen based on initial experiments.

The Central Composite experimental design is shown in Table 1The data was evaluated using Design- Expert® 7.1 software (Trial Version).

Twenty experiments were carried out using suggested ratio and parameters as per Central Composite model and values of the desired responses were obtained from the software. All the values were added in the software and Response Surface Methodology was used for optimization of the responses which in turn will help in mobile phase optimization with a suitable combination suggested by the systematic experimentation approach.

Following steps were performed in response Surface Methodology

1. Evaluation of the Model with help of ANOVA test to check the fitness of the model and to get an idea about important factors based on p values.
2. Optimization of the model can be performed graphically or numerically both approaches were tested in development process.
3. Optimization of model gives list of possible solution with desirability value. The solution with high desirability value was selected for practical applications.

Table 1. Central composite model with 3 factors

Run	F 1	F 2	F 3
	A:ACN%	B:pH	C:Flow Rateml/min
1	75.00	5.6	1.0
2	80.00	3.0	1.2
3	70.00	3.0	1.2
4	75.00	4.0	1.0
5	75.00	4.0	1.0
6	75.00	4.0	1.0
7	83.50	4.0	1.0
8	70.00	5.0	1.2
9	80.00	5.0	1.2
10	75.00	4.0	1.0
11	75.00	2.3	1.0
12	70.00	5.0	0.8
13	66.50	4.0	1.0
14	75.00	4.0	1.3
15	80.00	5.0	0.8
16	80.00	3.0	0.8
17	75.00	4.0	0.6
18	75.00	4.0	1.0
19	70.00	3.0	0.8
20	75.00	4.0	1.0

2.7 Stress Degradation Studies [40-44]

As the basic objective of the method development was development of a stability indicating assay method both drug samples were subjected to stress degradation conditions there

were very few references available at the time of development of this method based on that a systematic way was followed for performing stress degradation of the drugs. Both drugs were subjected to stress studies in Acidic, Alkaline, Neutral, Oxidative, Photo stress degradation along with dry heat stress conditions.

2.8 Stress Degradation under Acidic Environment

To check stability of the drugs in acidic environment both the drugs were subjected to acid treatment.Methanolic drug solution was mixed with 0.1 m Hydrochloric acid in equal proportion and the resulting solution was refluxed for 8 hours. After 8 hours solution was neutralized and then diluted to get a concentration of 10 mcg/ml for drug. These diluted solutions were injected in the system and using optimized mobile phase, stability of drugs towards acidic environment was observed.

2.9 Stress Degradation under Alkaline Environment

Stability of both drugs in alkaline medium was observed by treating methanolic drug solutions with .1 M sodium hydroxide in equal proportion.These solutions were refluxed for 8 hours and resulting solutions were neutralized and diluted to get desired concentration of 10mcg/ml.Diluted stress samples were injected in the chromatographic system to understand effect of alkaline environment on drug molecules.

2.10 Stress Degradation under Neutral Environment

A drug molecule can also undergo degradation at neutral pH value. Methanolic drug solutions were mixed with equal amount of double glass distilled water and the resulting solution was refluxed for 8 hours at 80^0C.The resulting solutions were diluted and were tested with the optimized mobile phase to study effect of neutral environment on stability of drug molecules.

2.11 Stress Degradation under Oxidative Environment

Many drug candidates are very much prone to degradation in oxidative conditions, to observe the effect of oxidative environment methanolic drug solutions were treated with 3% hydrogen peroxide solution and the resulting solution was refluxed for 8 hours at 80^0C. Resulting solution was diluted to get suitable concentration and was injected in the system and effect of oxidative stress condition was studied in both drug molecules.

2.12 Photo Degradation Study

Photo catalytic degradation is observed in many drug candidates owing to their chemical structure. In order to study effect of light on drug sample, methanolic solutions were exposed to sunlight on a bright sunny day and at the end of the day solution was refrigerated and on next day it was brought to room temperature and then again it was exposed to direct sunlight.(Guidelines suggest use of UV lamp with definite power to check this effect) but there are few references where in direct sunlight was used to study photo stability of drug molecule. The resulting solution was diluted to 10 mcg/ml and then solutions were observed for any change in chromatogram.

2.13 Stress Degradation under Dry Heat Conditions

In order to check thermal stability of drug moles standard samples of drug were placed in a petri plate and it was placed in a hot air oven for 8 hours at a temperature of 100^0C.The exposed powder was the transferred to a volumetric flask and it was diluted with methanol to get concentration of 10mcg/ml.
SystemSuitability parameters are specified in USP value of these parameters is expressed after analyzing samples in triplicate

2.14 Validation of Developed HPLC Method [45-46]

With reference to regulatory requirements of ICH guidelines it is essential to validate any analytical method for its intended purpose. Various validation parameter includes Accuracy, Linearity, Limit of Detection, Limit of Quantitation, Selectivity and Robustness and Ruggedness.

3. RESULTS

Response surface methodology was used for the optimization of chromatographic parameters. After initial 20 experimental runs as per Central Composite Design values of all response variables were entered in the software and further analysis of significant factors and

interdependent terns were identified based on ANOVA test for a quadratic model and significance of models were determined based on lower p value. Model p value and lack of fit values were used for determining the significance of each factor.

The magnitude of the coefficients in the equations for the responses and the lower p-value (<0.001) indicated that percentage of organic phase, pH of the buffer and flow rate significantly affected the responses.

The optimum conditions were calculated using numerical optimization.

To achieve the composite desirability (di), the response criteria were set as per requirements of the method for the responses the importance factors for all responses were set as 3+. Derringer's desirability was calculated, and the optimum solution was determined to be a percentage of organic phases ACN 75%, the desirability graph indicated that the maximum desirability was achieved for the pH of 4.0 and at a flow rate of 1.0 ml/min.

To confirm the point prediction values, experiments (n = 3) were conducted to determine the mean responses R1S, R2 S and Rt. The experimental results were found to be R1 S experimental = 2.05, R2 S experimental = 4.21 and Rt experimental = 12.98, representing good agreement with the predicted results.

Four Solutions were found from the design experiments Derringer's desirability was used as evaluating tool results are shown in Table 2.

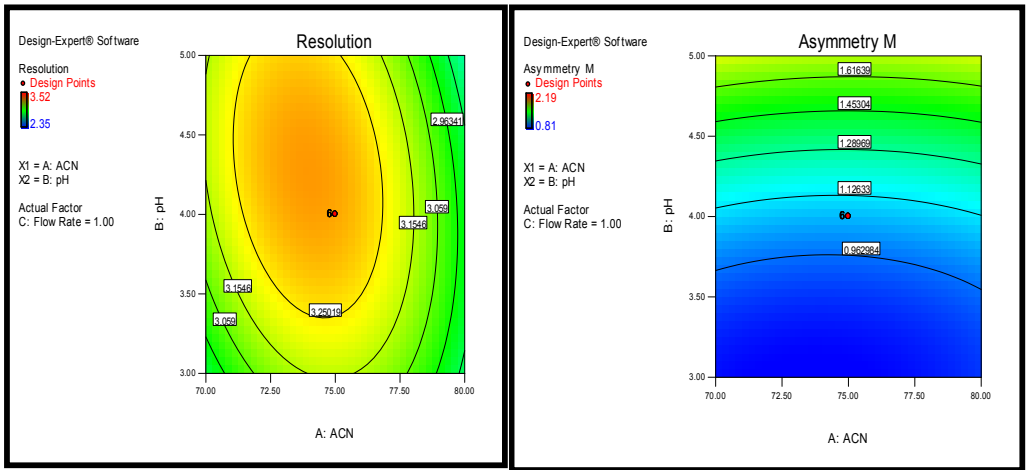

Fig. 1. Response surface plot for resolution and Asymmetry for Rupatadine

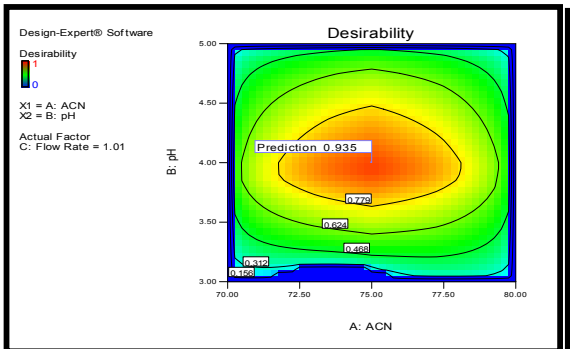

Fig. 2. Desirability Graph

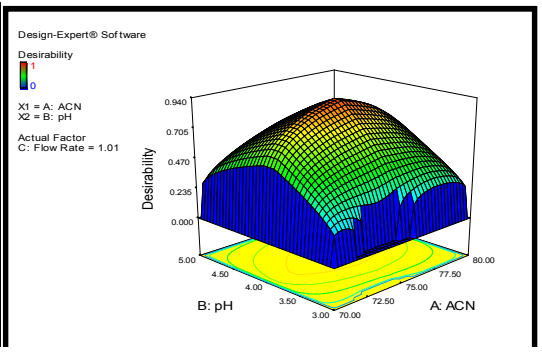

Fig. 3. Designs for MKT and RUPA by DOE

Table 2. Solutions from the design experiments Derringer's desirability

Sr.No	ACN	pH	Flow Rate	Res	Asy.M	Asy R	Rt M	Rt R	Desirability
1	75	4	1.01	3.3252	1.0615	1.6409	13.2034	4.077772	0.935306
2	75	4	1.01	3.3246	1.0615	1.6410	13.20038	4.074179	0.935303
3	75	4	1.01	3.3258	1.0615	1.640	13.20663	4.081619	0.935302
4	75	3.98	1.01	3.3235	1.0539	1.6338	13.1991	4.073021	0.935219

3.1 Solubility of Drugs in Different Solvents

Solubility pattern of both drugs was studied by dissolving them in different solvents. Montelukast was freely soluble in methanol and water, whereas Rupatadine is soluble in methanol.

Initially a mobile phase was optimized for identification of both drugs , but when same mobile phase was used for determination of stability indicating nature of the method, separation of degradation products from pure drug peak resolution was not sufficient and some degradation products were co eluting near retention time of the drugs, in order to solve this issue a new technique of Design of experiments was used and based on previous knowledge central Composite Design was constructed and similar experiments were carried out based on the responses selected an response surface methodology tool was used for optimization of mobile phase according to requirements for separations.

Suggested solution from DoE experiment was used to check applicability of it for explaining stability indicating nature of the method.

It can be observed that the newly designed mobile phase and chromatographic parameters gives desired runtime and sufficient resolution in order to use it as a stability indicating method.

3.2 Evaluation of Analytical Method (Method Validation)

Developed analytical proposed methods were validated as per guideline laid by the International Conference on Harmonization (ICH) procedure and United State Pharmacopoeia 24 (USP 24).

3.3 Linearity and sensitivity (Limit of Detection and Limit of Quantitation)

Linearity shows the direct relationship of response to the concentration of analyte, it was evaluated by constructing a calibration curve of concentration against peak area for both the drugs separately. Linearity was expressed in terms of correlation coefficient, and it was found to be 0.9996 and 0.997 for Montelukast and Rupatadine respectively. These values indicate good correlation between response and concentration and method follows linearity. Values gained for the selected calibration curve and their related validation parameters are shown in TableS 4,5 and 6. Calibration graph of Montelukast Sodium and Rupatadine Fumarate are shown in Figs 5 and 6 respectively.

Limit of detection and limit of quantitation were determined based on the calibration curve data. It was found that minimum concentration detected by the method was 4.06 mcg/ml for Montelukast where as it was found to be 4.10 for Rupatadine fumarate. Minimum quantitation limit was found to be 12.13 mcg/ml and 13.12 mcg/ml for Montelukast and Rupatadine respectively.

Precision: Precision, reproducibility and accuracy study of the proposed approach were judged by performing replicate investigation of the working standard solutions. Within the linearity calibration curves, the selected concentrations were prepared and analyzed with the developed method to estimate the Intra-day and Inter-days variability. For the Intra-day analysis repeated injection of the selected solution were assessed on the same day. Whereas, Inter-days study were continued five injections were completed for three consecutive days. Precision and reproducibility were expressed in terms of as the % RSD, [Table 7]. Observation of the resulting values indicates that developed method shows good inter day and Intraday precision.

Accuracy: To check the degree of accuracy of the method, recovery studies were performed in triplicate by standard addition method at 3 levels of 50%, 100% and 150%. Known amounts of standard Montelukast sodium and Rupatadine were added to pre-analyzed formulation samples separately and were subjected to the HPLC analysis using optimized parameters.

Results of recovery studies are shown in Table 8.

Mean percentage recovery of the analyte was estimated by comparing the concentration obtained from spiked analyte with the actual added concentration. It was observed that mean recovery for both drugs was within 98-102% limit.Also, recovery study outcomes exposed the absence of interference from commonly encountered pharmaceutical excipients present in the selected formulation.

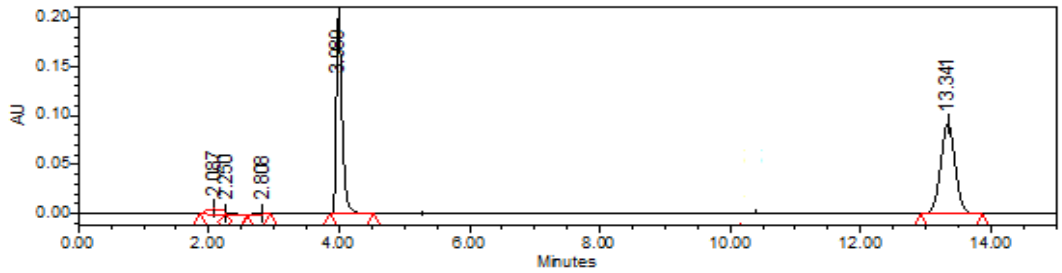

Fig. 4. Chromatogram of standard montelukast and rupatadine fumarate with DoE

Table 3. System suitability test parameter for montelukast and rupatadine (n =5).

Parameter	Montelukast sodium	Rupatadine fumarate
Retention Time (min) ± %RSD	3.99±2.0124	13.25±1.564
Tailing Factor ± %RSD	1.03±0.4587	1.11±1.0256
Theoretical Plates ± %RSD	4259±2.4125	5538±2.2154
Resolution ± %RSD	1.87±1.06	1.96±0.798

Table 4. Linearity of montelukast sodium

Sr. No.	Concentration	RT	Peak area	Area %
1	(10µg/mL)	13.0471	7612786.01	99.76
2	(20µg/mL)	13.0531	14795030.02	99.84
3	(30µg/mL)	13.0670	23341852.05	99.90
4	(40 µg/mL)	13.0701	31573265.15	99.93
5	(50 µg/mL)	13.0970	39138953.45	99.92
6	(60 µg/mL)	13.0802	46661997.52	99.71
Mean		**13.0694**	**27520647.25**	**99.84**

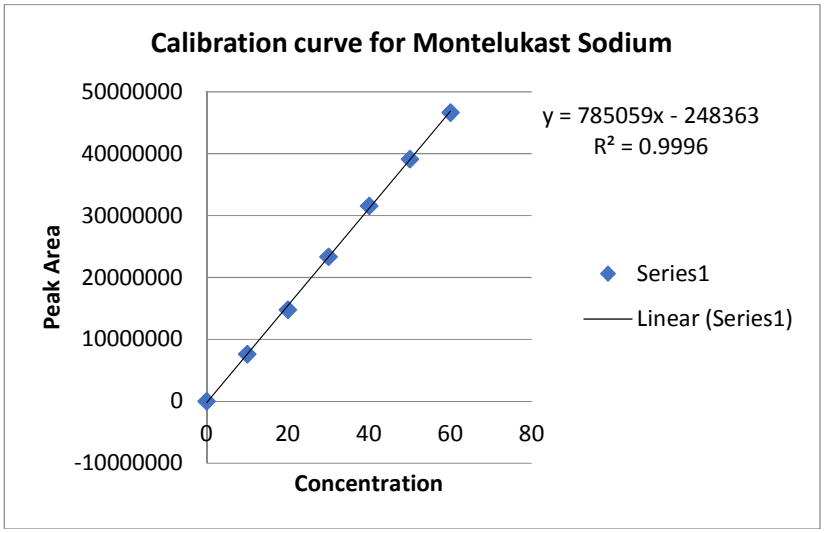

Fig. 5. Calibration curve of montelukast sodium

Table 5. Linearity of rupatadine fumarate

Sr. No.	Concentration	Rt	Peak area	Area %
1	10µg/mL	4.1501	6103634.10	100
2	20µg/mL	4.1705	9930299.01	100
3	30µg/mL	4.1730	17039417.02	100
4	40 µg/mL	4.1770	23165269.03	100
5	50 µg/mL	4.1871	28306925.11	100
6	60 µg/mL	4.1805	34162443.12	100
	Mean	**4.1728**	**19784666.12**	**100**

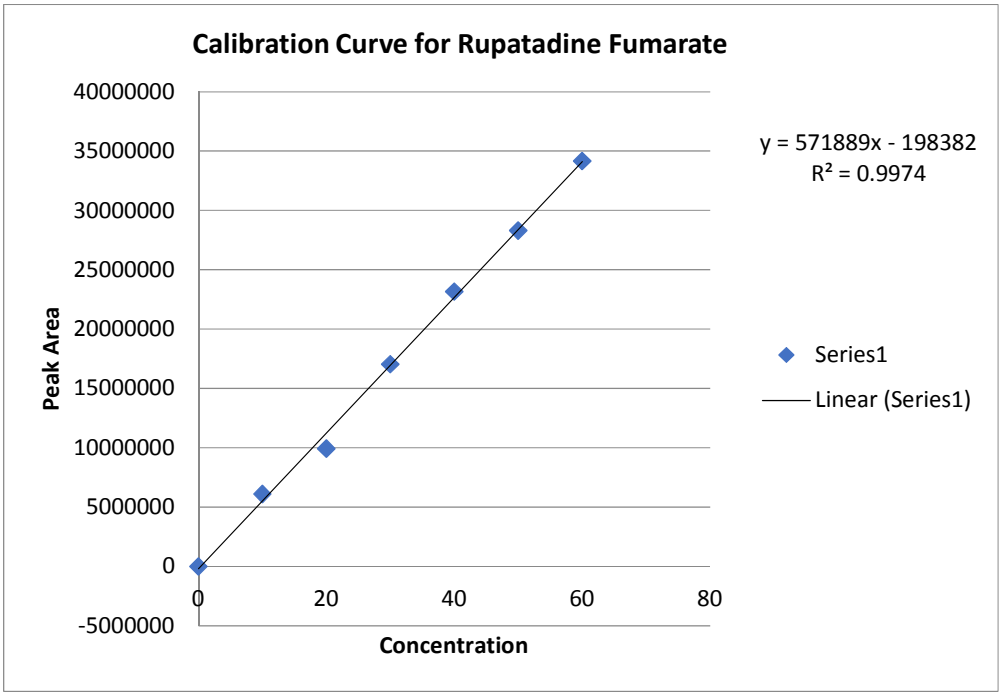

Fig. 6. Calibration plot of rupatadine fumarate

Table 6. Details of the linear regression analysis of Montelukast and Rupatadine

Parameter	Montelukast sodium	Rupatadine fumarate
Linearity range (µg/ml)	10-50	10-60
Correlation coefficient (r^2)	0.9997	0.9974
Detection limit (µg/ml)	4.0615	4.1020
Quantification limit (µg/ml)	12.1315	13.1245

Table 7. Interday and intra day precisionfor Montelukast and Rupatadine

Concentration i(µg imL^{-1})	MKT				RUPA			
	Intra Day Measured Concentration		Inter Day Measured Concentration		Intra Day Measured Concentration		Inter Day Measured Concentration	
	Mean	RSDi%	Mean	RSDi%	Mean	RSDi%	Mean	RSDi%
10	10.15	1.4515	10.78	0.6554	10.65	0.1501	10.61	0.1457
30	30.45	0.9542	30.01	0.4631	30.05	1.1553	30.04	1.5311

Robustness: Robustness shows the ability of the method to remain unaffected even after small but deliberate alteration in chromatographic parametersDeliberate changes were made insome chromatographic parameters namely flow rate, wavelength and the ratio of mobile for the developed methods for Montelukast sodium and Rupatadine. The results are showed in Tables 9 and10.It was observed that even after small alterations in the experimental parameters no significant changes in the results obtained after sample injection.

Table 8. Recovery studies Montelukast and Rupatadine

Tablet Sample							
Preanalyzed Formulation (µg/ml)		Amount of standard drug Spiked(µg/ml)		Pure Drug recovered (µg/ml)		% Recovery	
MKT	**RUPA**	**MKT**	**RUPA**	**MKT**	**RUPA**	**MKT**	**RUPA**
10	10	5	5	15.03	14.862	100.2	99.08
10	10	10	10	15.23	14.71	101.533	98.066
10	10	15	15	15.11	14.82	100.733	98.8
Mean				15.50	14.00	100.45	15.123
SD				0.3573	0.5348	2.8700	0.1006
% RSD				2.8572	0.2771	2.8571	0.6656

Table 9. Robustness of Montelukast Sodium

Parameters	R.time	Peak Area	%Area	TPlates	Asymmetry
Flow Rate					
Flow Rate	13.833	9304233	99.96	5307	1.07
0.8 mL/min.	13.813	9416908	99.85	5312	1.09
	13.810	9387952	99.76	5323	1.06
Mean	13.817	9369697.67	99.85	5314	1.0733
STDEV	0	58513.4	0.10	8.185352772	0.0152
%RSD	0	0.6244	0.10	0.1540	1.4231
Flow Rate	11.753	7484951	99.97	3816	1.19
1.2mL/min.	11.745	7554962	99.89	3798	1.17
	11.753	7410276	99.99	3805	1.16
Mean	11.7503	7483396.33	99.95	3806.33	1.1733
STDEV	0.0046	72355.5277	0.0529	9.0737	0.0152
%RSD	0.2638	0.9668	0.0529	0.2383	1.3018
Wavelength					
Wavelength	13.117	8319866	99.72	4522	1.098
244nm	13.117	8284745	100	4544	1.074
	13.142	8221132	99.88	4452	1.08
Mean	13.1253	8275247.67	99.866	4506	1.084
STDEV	0.0144	50047.479	0.1404	48.041	0.0124
%RSD	0.6791	0.6047	0.1406	1.0661	1.1522
Wavelength	13.12	6951486	99.66	4598	1.10
248nm	13.11	7022558	100.00	4575	1.07
	13.12	6921585	100.35	4558	1.07
Mean	13.11	6965209.7	100.00	4577	1.08
STDEV	0.0058	51866.56	0.3430	20.0749	0.0156
%RSD	0.0440	0.7447	0.3430	0.4386	1.4463
Mobile phase					
Mobile phase	12.07	76824589	99.25	4256	1.13
68-32	12.27	76830833	98.56	4199	1.12
	12.08	77414627	100.01	4192	1.14
Mean	12.14	77023349.7	99.28	4215.667	1.13
STDEV	0.1124	338870.4923	0.7243	35.1046	0.0100
%RSD	0.9263	0.4400	0.7295	0.8327	0.8850
Mobile phase	13.35	6955145	99.45	4570	1.08
72-28	13.5	7027564	100.21	4568	1.09
	13.15	6921545	99.45	4578	1.07
Mean	13.33	6968084.7	99.70	4572	1.08
STDEV	0.1756	54181.0	0.4408	5.2915	0.0080
%RSD	1.3170	0.7776	0.4421	0.1157	0.7421

Table 10. Robustness of Rupatadine fumarate

Adjusted Parameters	Retention time	Peak Area	%Area	Theoretical Plates	Asymmetry
Flow Rate					
Flow Rate	5.073	32306925	100	8012	1.188
0.8mL/min.	5.073	32057588	100	7868	1.215
	5.077	32259732	100	7922	1.202
Mean	5.0743	32208081.7	100	7934	1.201
STDEV	0.0023	132450.189	0	72.746	0.0135
%RSD	0.0455	0.4112	0	0.9168	1.123
Flow Rate	3.38	24356549	100	6161	1.716
1.2mL/min.	3.383	24718933	100	6252	1.725
	3.383	24869763	100	6259	1.686
Mean	3.382	24648415	100	6224	1.709
STDEV	0.0017	263774.039	0	54.6717	0.0204
%RSD	0.0512	1.0701	0	0.8784	1.1948
Wavelength					
Wavelength	4.047	29639521	100	6558	1.335
244nm	4.05	29905738	100	6697	1.352
	4.057	29299609	100	6628	1.33
Mean	4.0513	29614956	100	6627.66	1.339
STDEV	0.0051	303810.2	0	69.500	0.0115
%RSD	0.1266	1.0258	0	1.0486	0.8612
Wavelength	4.05	27287866	100	6172	1.209
248nm	4.051	27783218	100	6145	1.22
	4.053	27635542	100	5989	1.211
Mean	4.0513	27568875.3	100	6102	1.2133
STDEV	0.0015	254316.2	0	98.7876	0.0058
%RSD	0.0377	0.9224	0	1.6189	0.4829
Mobile phase					
Mobile phase	4.187	28706244	100	9062	1.21
68-32	4.137	29116424	100	9053	1.18
	4.167	28514126	100	9041	1.2
Mean	4.1636	28778931.3	100	9052	1.1966
STDEV	0.0251	307657.7	0	10.5356	0.0152
%RSD	0.6044	1.0690	0	0.1163	1.2764
Mobile phase	4.113	28790925	100	9153	1.15
72-28	4.213	28250478	100	9168	1.14
	4.163	28514565	100	9179	1.16
Mean	4.163	28518656	100	9166.66	1.15
STDEV	0.05	270246.7	0	13.0511	0.01
%RSD	1.2010	0.9476	0	0.1423	0.8695

3.4 Analysis of the Marketed Formulation

Optimized mobile phase was used for analysis of pharmaceutical formulation.

Linear regression equation was employed to estimate the amount of Rupatadine and Montelukast tablet. The amount found is calculated which was found to be within the limit of label claim as mentioned in Table 11.

4. STABILITY INDICATING NATURE OF THE METHOD

4.1 Stress Degradation STUDIES

A stock solution containing 100 mg of drug in 100 ml methanol get final concentration 1mg $mL^{-1}$was prepared. This solution was used for force degradation to provide an indication of stability indicting property of the proposed method.

4.2 Study of Acid Induced Degradation Product

To check the effect of acid hydrolysis, acid degradation product was injected in the system. Interference of blank Hydrochloric acid was studied by injecting 0.1 M HCl in the system Fig. 8.

Careful observation of blank and degradation chromatogram shows that an additional peakswere present at 6.18 min, 8.79 min and 10.48 min along with drug peak [Fig. 9] in acid

induced hydrolytic conditions for Montelukast Sodium which shows that Montelukast is prone to acid degradation three degradation products were formed in the process of acid hydrolysis. Drug peak showed less than 10% degradation.

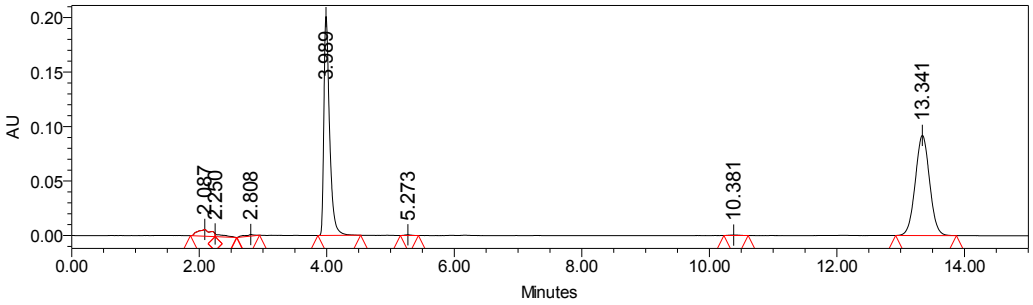

Fig. 7. Chromatogram of marketed formulation

Table 11. Analysis of Marketed Formulation

Sr. No.	Labeled Claim (mg/ ml)		Total amount recovered (mg/ ml)		% Label claim	
	Montelukast Sodium	Rupatadine fumarate	Montelukast Sodium	Rupatadine fumarate	Montelukast Sodium	Rupatadine fumarate
1	10	10	9.845	10.107	98.45	101.07
2	10	10	10.04	10.042	100.4	100.42
3	10	10	9.68	9.863	96.8	98.63
Mean			9.855	10.004	98.55	100.04
SD			0.1802	0.1263	1.8020	1.2636
% RSD			1.8285	1.2631	1.8285	1.2631

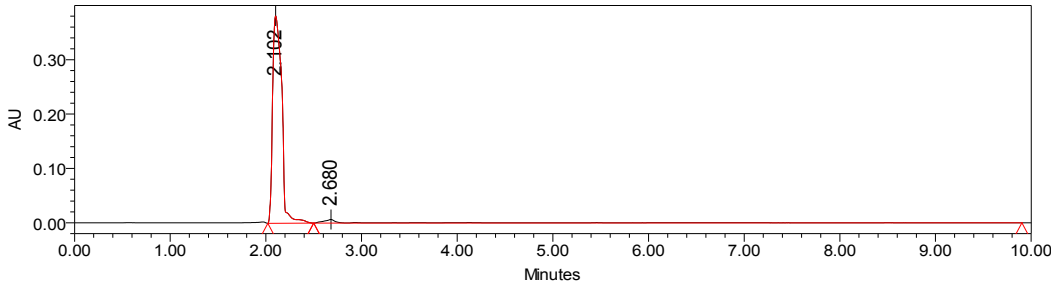

Fig. 8. Chromatogram of Blank Hcl

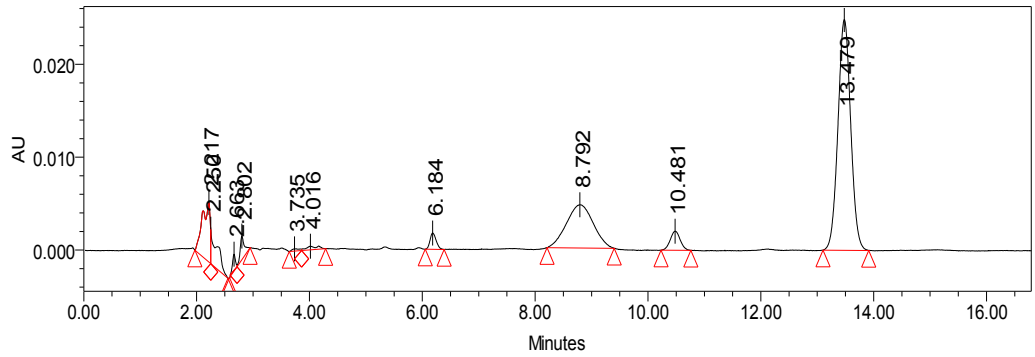

Fig. 9. Chromatogram of acid induced hydrolytic sample of Montelukast sodium

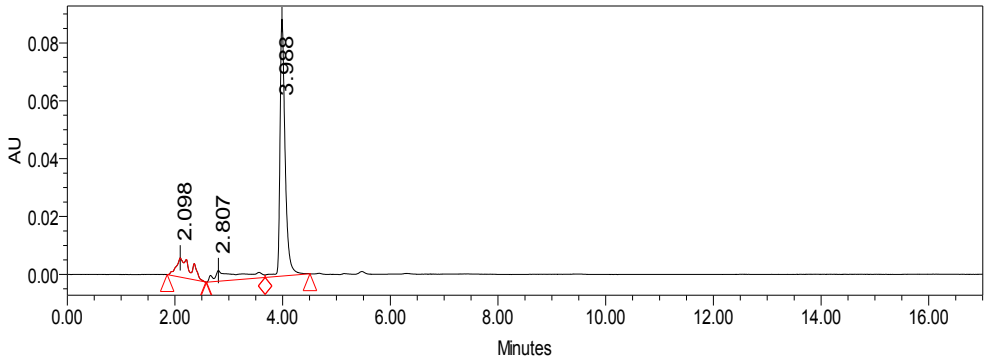

Fig. 10. Chromatogram of acid hydrolysis of Rupatadine fumarate

When Rupatadine sample was treated in acidic environment no additional peaks were observed in the chromatogram suggesting stability of Rupatadine in acidic environment [Fig.10].

4.3 Alkali induced Hydrolysis

Initially Blank is injected in the system chromatogram of blank is shown in Fig. 11.

Alkali induced sample of Montelukast sodium shows additional peak at 6.181 min and 10.45 min along with drug peak indicating formation of additional degradation [47-48] products in the alkaline environment and drug is prone to undergo degradation in alkaline conditions. Chromatogram is shown in Fig.12.

Rupatadine fumarate sample which was degraded in alkaline conditions was chromatographed using same optimized mobile phase chromatogram is shown in Fig. 13.

The chromatogram of Rupatadine fumarate does not show additional peak in the alkaline environment suggesting stability of the drug in alkaline conditions.

4.4 Degradation under Neutral Conditions

Neutral hydrolysis of both the drugs does not show any additional peaks in the chromatogram.

Preparation of Hydrogen Peroxide induced degradation product: Peroxide induced degradation sample of Montelukast sodium was analyzed by optimized mobile phase.

It was observed that chromatogram of stress sample shows additional peak at 5.13 and 5.496minalong with drug peak which show that drug is prone to oxidative environment, and it undergoes degradation forming two degradation products. Chromatogram is shown in Fig. 15.

Rupatadine fumarate sample was analyzed and chromatogram was shown in Fig.16.
No additional peaks were observed in the chromatogram of Rupatadine in oxidative environment in case of Rupatadine Fumarate.

Photo-degradation product: It was observed that Montelukast chromatogram shows additional peaks at 5.03 min and 10.25 min along with drug peak. When drug was exposed for lesser period of time even then similar peak were obtained after2 hour exposure which shows that Montelukast is undergoing photo catalytic degradation very fast in direct sunlight.

When Rupatadine was exposed to sunlight it does not showed any additional peak in the chromatogram, this shows that drug is very much stable in most of the stress conditions, To study the stability indicating nature of developed method all the degradation samples were mixed together and resulting solution was injected in the HPLC system It was observed that all degradation peaks are well resolved in case of Montelukast sodium. When all degradation samples are mixed and injected in the system no additional peaks were observed in which shows stability of Rupatadine in all stress conditions.

These chromatograms of all impurities of Montelukast sodium are resolved properly.

When all stress samples of both drugs were mixed and injected in the HPLC system and optimized chromatographic conditions were used, the resulting chromatogram shows very

well resolved degradation peaks and drug peaks in same chromatogram. This observation shows stability indicating behavior of the method in which all the degradation products of Montelukast and Rupatadine are resolved, and drug peaks can be seen separately, and this method can be used for routine analysis of the drugs in combined dosage form.

Comparison of merits over reported stability indicating method and developed method are shown in Table 12.

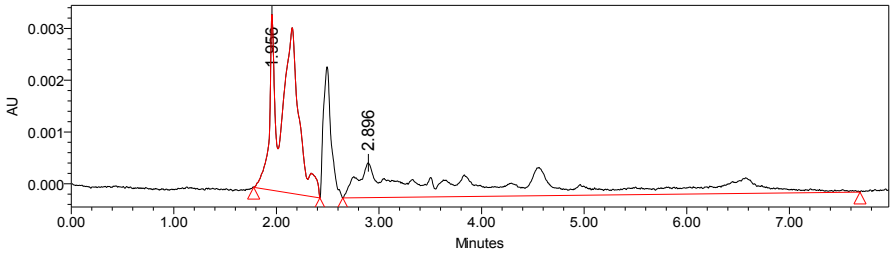

Fig.11. Chromatogram of Blank NaOH

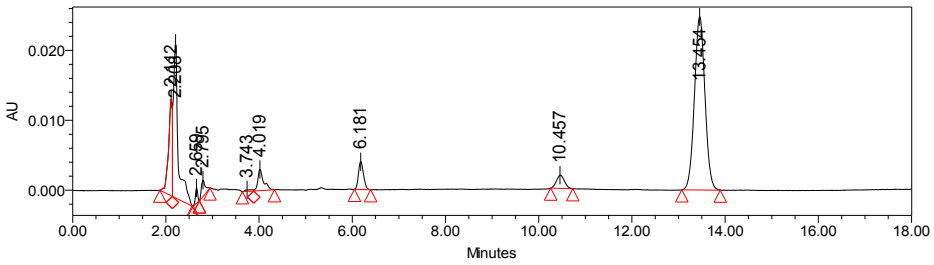

Fig. 12. Chromatogram Alkali induced sample of Montelukast sodium

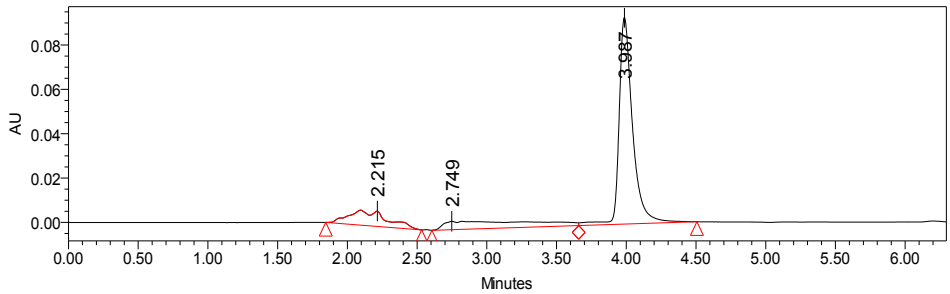

Fig. 13. Chromatogram Alkali induced sample of Rupatadine Fumarate

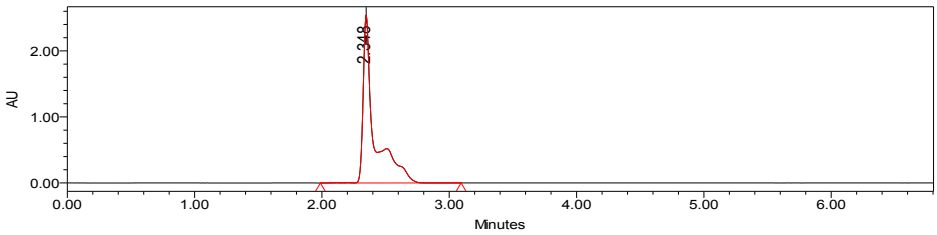

Fig. 14. Chromatogram of Blank Hydrogen Peroxide

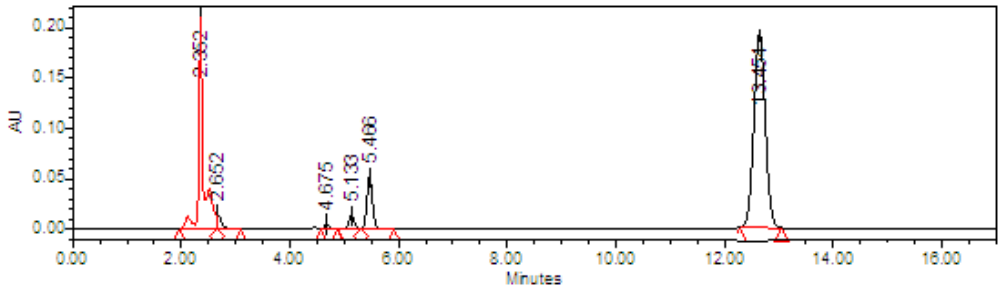

Fig. 15. Chromatogram of Peroxide induced degradation sample of Montelukast sodium

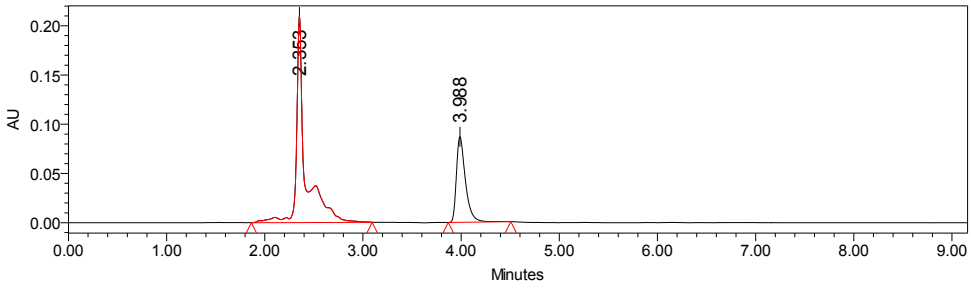

Fig.16 Chromatogram of Peroxide induced degradation sample of Rupatadine

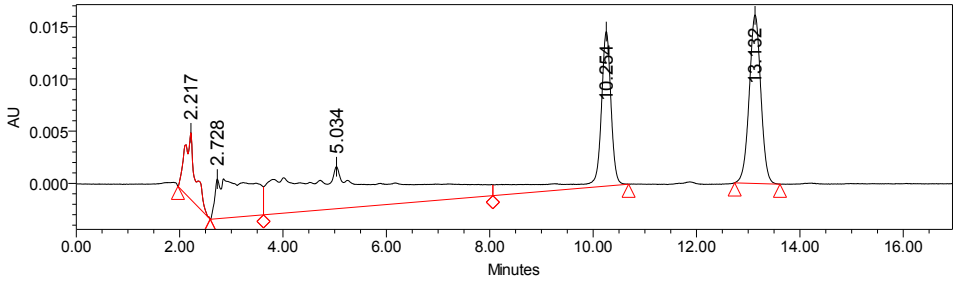

Fig. 17. Chromatogram of photo degradation induced sample of Montelukast in direct sunlight

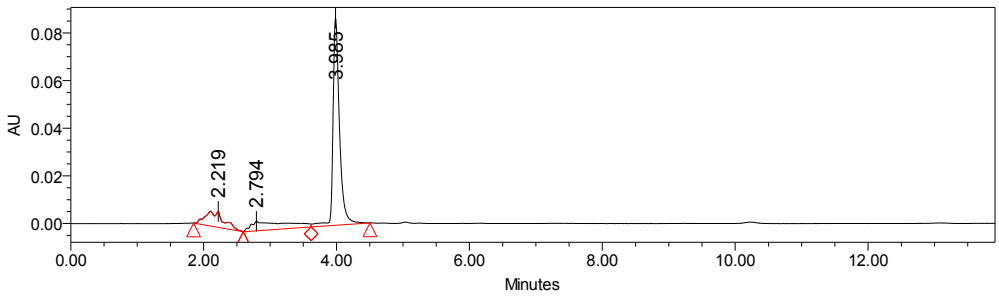

Fig. 18. Chromatogram of photo degradation induced sample of Rupatadine Fumarate in sunlight

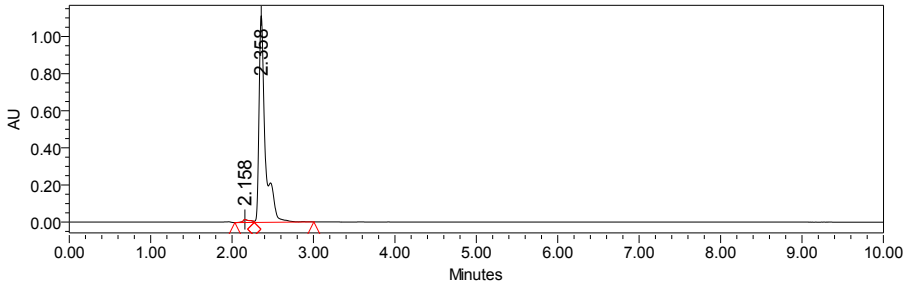

Fig. 19. Chromatogram of solvent used for sample preparation

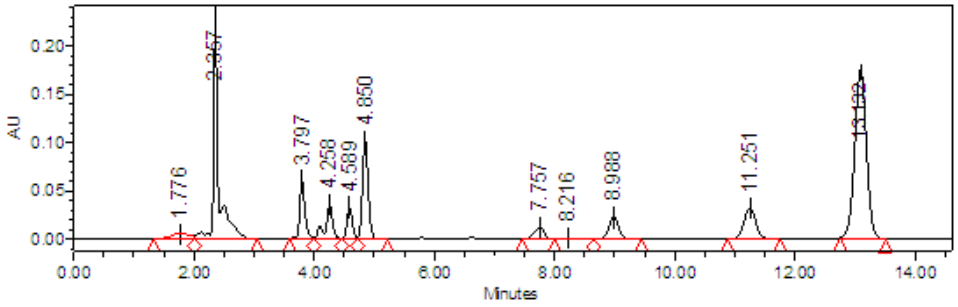

Fig. 20. Chromatogram of mixed stress samples for Montelukast

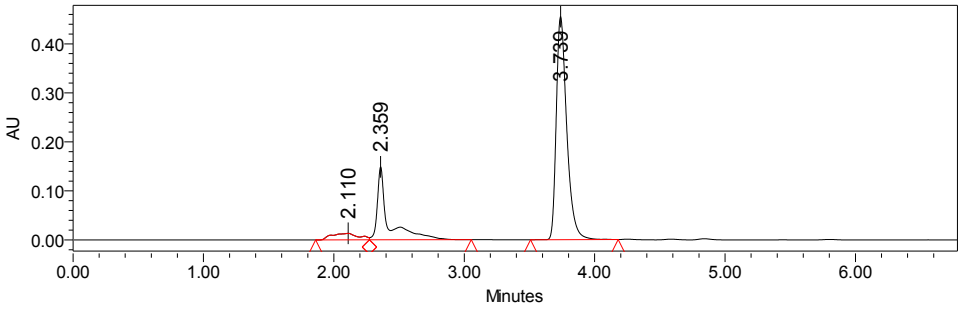

Fig. 21. Chromatogram of mixed stress samples for Rupatadine

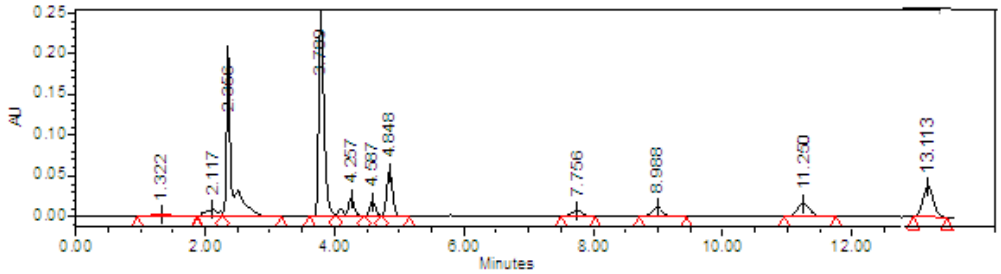

Fig. 22. Chromatogram of mixed stress samples for Montelukast and Rupatadine

Table 12. Merits over reported stability indicating method

Sr. No.	Reported Stability Indicating Method	New Stability Indicating method
1	Developed with traditional technique	Developed and optimized with Design of experiment technique.
2	pH of buffer is 3.0	pH of buffer is 4.0 suitable for silica columns.
3	Runtime is more than 15 mins	Run time is less than 15 mins
4	Methanol: acetonitrile: buffer (40 : 30 : 30 v/v) as mobile phase	Acetonitrile: Buffer (75:25v/v) as mobile phase.

5. DISCUSSION AND CONCLUSION

The proposed approach of design of experiment in design, optimization and development of stability indicating HPLC method is accurate, precise, fast and selective for the simultaneous estimation of Montelukast sodium and Rupatadine fumarate in bulk and solid dosage form. The method can be used as a stability indicating method as degradation products are resolved from the drug peaks. The proposed assay outcomes, recovery value for the selected tablets were in good accord with their respective labeled claims Non-interference of the excipients was observed through the analytical run which shows accuracy of the method. All the results of different validation parameters were found to be with in specified limits of regulatory guidelines. Hence this method can be conveniently adapted for the routine quantitative estimation and studying stability indicating nature of drugs.

DISCLAIMER

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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