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A VALIDATED STABILITY-INDICATING AND LC-MS COMPATIBLE METHOD FOR THE DETERMINATION OF RELATED SUBSTANCES AND ASSAY OF MONTELUKAST SODIUM AND LEVOCETIRIZINE HCL BY U-HPLC IN TABLET DOSAGE FORM

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Abstract:
A novel gradient reverse-phase ultra-high performance liquid chromatography (U-HPLC) method has been developed and validated for determination of related substances and assay of Montelukast sodium and Levocetirizine HCl in 4 mg Montelukast sodium and 2.5 mg Levocetirizine HCl tablet. The separation of both drugs from their related impurities has been successfully achieved using Kinetex, Phenyl-Hexyl (100°A,150x4.6 mm, 5µm) column maintained at 20ºC±2ºC. Flow rate was optimized at 1.2 ml/min. The wavelength was fixed at 230 nm with injection volume of 20 µl. Sensitivity, linearity, establishment of relative response factor, limit of quantitation and forced degradation were performed as part of suitability of the method. The gradient programme of this method was checked in mass analysis of all impurities and both drugs as U-HPLC parameter with mass parameters to study the compatibility of the method in mass analysis. Assay method was also developed using same column, flow rate and injection volume. But here, runtime was shorter with higher column oven temperature (30ºC±2ºC). Specificity, accuracy, linearity, precision and solution stability of standard, sample and mobile phase were determined as part of validation. Filter variability in assay method was evaluated through determining similarity factor between assay values of unfiltered and PVDF with nylon.

Key Words: *Reverse-phase U-HPLC, gradient programme, related substances, LC-MS, assay, method development, validation*

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INTRODUCTION:

Now a day, Allergic Rhinitis is a global health issue. It is responsible for major illness and disability. About 10-20% of population is affected by this disease throughout the world. The main symptoms are nasal congestion, rhinorrhoea, itching, and sneezing [1]. Levocetirizine, the active R-enantiomer of cetirizine, is a potent third generation histamine (H1) receptor antagonist effective against persistent allergic rhinitis [2]. Montelukast, type I receptor antagonist of leukotriene D4, has both anti-inflammatory and bronchodilator properties. The combined therapy of Montelukast sodium with Levocetirizine HCl provides enhanced and complimentary effects thereby reducing the symptoms effectively [3].

In past, no such article has been published on quantification of related substances in mass-compatible method along with individual drug in this combined therapy. Charde et al have developed and validated a reverse-phase high performance liquid chromatographic (HPLC) method for estimation of related substances of Montelukast from Montelukast Sodium chewable tablets [4]. They used BDS Hypersil C18 (250mm x 4.6mm, 5µ). Buffer and acetonitrile in the ratio of 30:70 were used for this separation. This method was validated with respect to specificity, linearity, accuracy, precision and ruggedness. V.K. Akula, B.N. Sinha and H.J. Seok have developed a stability indicating gradient HPLC method for determination of related substances in Levocetirizine HCl oral solution [5]. The separation was achieved using Kinetex biphenyl (250mm x 4.6mm, 5 µ) column. In this gradient programme, sodium perchlorate in water and acetonitrile were two mobile phases eluted in different ratio with respect to time. Wavelength was set at 230 nm. Under method validation, precision, accuracy, linearity & range, robustness, determination of limit of detection and quantitation were performed.

The present study was carried out to find a suitable stability indicating reverse-phase U-HPLC method for estimation of related substances in combined formulation of Montelukast Sodium [sodium; 2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl) ethenyl] phenyl]-3-[2-(2-hydroxypropan-2-yl) phenyl] propyl] sulfanylmethyl] cyclopropyl] acetate] and Levocetirizine HCl [2-[2-[4-[(R)-(4-chlorophenyl)-phenylmethyl] piperazin-1-yl] ethoxy] acetic acid; dihydrochloride] shown in Figure 1 & 2. Acid & base degradation, oxidation, hydrolysis, thermal degradation, photolytic (exposure to UV light and LUX light) degradation studies were performed to evaluate the percentage of impurities generated. Both drugs and their impurities were listed in Table 1. Establishment of relative response factor (RRF) for individual impurity, determination of linearity and limit of quantitation (LOQ) were also established. Robustness of the developed method through change in flow & column oven temperature was assessed too. This related substance method was applied in Mass analysis of both drugs along their impurities as U-HPLC part to evaluate the compatibility of the method in mass analysis. Analytical method for assay of both drugs was also developed for simultaneous determination. Accuracy, precision and linearity were also conducted to evaluate this analytical method. In addition, solution stability of standard, sample and mobile phase were determined. Filter variability was tested in assay method.

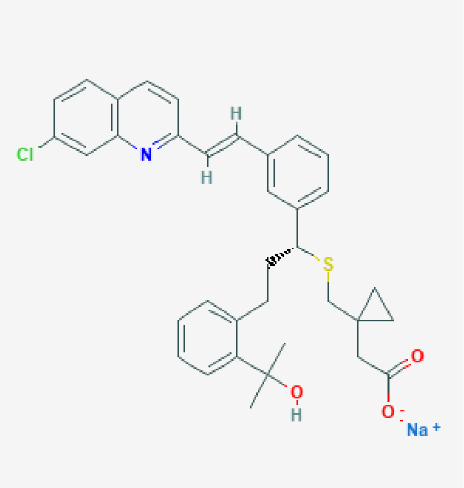


Fig. 1: Montelukast sodium

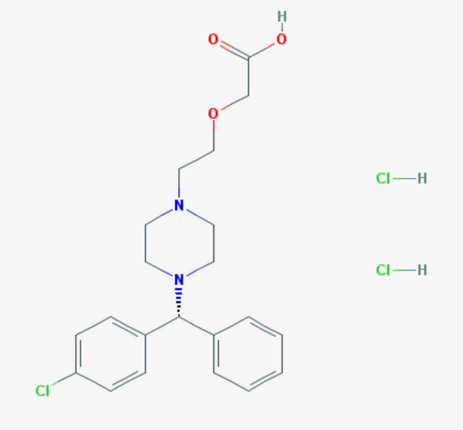


Fig. 2: Levocetirizine HCl

Table 1: List of Montelukast sodium & Levocetirizine HCl with their impurities, IUPAC name and molecular weight [6, 7, 8]

Name of Compounds	IUPAC Name	Molecular Weight
Montelukast	2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanylmethyl]cyclopropyl]acetic acid	586.187
Montelukast Sulfoxide	2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfinylmethyl]cyclopropyl]acetic acid	602.186
Cis-Isomer of Montelukast	2-[1-[[(1R)-1-[3-[(E)-2-(7-chloroquinolin-2-yl)ethenyl]phenyl]-3-[2-(2-hydroxypropan-2-yl)phenyl]propyl]sulfanylmethyl]cyclopropyl]acetic acid	586.187
Michael adduct	1-[[[(1R)-1-[3-[(1R)-1-[[[1-(Carboxymethyl)cyclopropyl]methyl]thio]-2-(7-chloro-2-quinolinyl)ethyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl) phenyl] propyl] thio]methyl]-cyclopropaneacetic acid	732.39
Montelukast Methyl Ketone	[1-[[[(1R)-3-(2-acetylphenyl)-1-[3-(E)-2-7-chloroquinolin-2-yl) ethenyl] phenyl] propyl] sulfanyl] methyl] cyclopropyl] acetic acid	570.144
Methyl Styrene	[1-[[[(1R-1-[3-[(E)-2-(7-Chloroquinolin-2-yl) ethenyl] phenyl]-3-[2-(prop-1-en-2-yl) phenyl] propyl] sulfanyl] methyl] cyclopropyl] acetic acid	568.172
Levocetrizine HCl	2-[2-[4-[(R)-(4-chlorophenyl)-phenylmethyl] piperazin-1-yl] ethoxy] acetic acid; dihydrochloride	388.892
4-Chloro Benzhydryl Piperazine	1-[(4-Chlorophenyl) phenylmethyl] piperizine	286.8
Amide Impurity	(R)-2-(2-(4-((4-Chlorophenyl)(phenyl) methyl) piperazin-1-yl) ethoxy) acetamide	387.9

MATERIAL AND METHODS:

Chemicals

Montelukast Sodium, Impurities of Montelukast sodium (Sulphoxide, Cis-Isomer, Michael adduct, Methyl Ketone and Methyl Styrene), Levocetirizine HCl, Impurities of Levocetirizine HCl (4-Chloro Benzhydryl Piperazine and Amide Impurity). Trifluoroacetic acid was purchased from JT Baker supplied by Avantor India. Acetonitrile, methanol, PDVF and Nylon filters were purchased from Merck (Germany).

Instrument and chromatographic condition in U-HPLC for Related Substances (Optimized method)

Agilent (Model: 1260 Infinity II & DAD) (U-HPLC) were employed connected to Empower 3.0 data integrator module. The separation was performed using an analytical column Kinetex, Phenyl-Hexyl (100°A, 150mm x 4.6mm, 5µ) maintained at 20ºC±2ºC. The wavelength was optimized at 230 nm. Flow rate was set at 1.2 ml/min with injection volume of 20 µl. 90% methanol in water was used as needle wash. Two different mobile phases were mixed in a proportionate way to run this gradient programme. One mobile phase consisted of water and trifluoroacetic acid in the ratio of 1000:1.5. Another mobile phase was mixture of acetonitrile and methanol in the ratio of 5100:900 followed by addition of 9 ml trifluoroacetic acid. At the beginning, both mobile phases were mixed at the ratio of 70:30. At 15 minute, ratio became 60:40 and at 28 minute, it changed to 50:50. Ratio again changed to 30:70 at 40 minute. Ratio became 20:80 at 45 minute. At 50 minute, ratio was returned to its initial position *i.e*. 70:30 and hold up to 55 minutes. So, it was run of 55 minutes.

Instrument and chromatographic condition in LC-MS (Optimized method)

The developed related substance method was followed here also in U-HPLC part. Waters Micromass (Model: Quattro micro API, Alliance 2695) was used for characterization purpose connected with Mass Lynx 4.1 data integrator module. Under mass acquisition method, full scan was selected. Measurement was performed under both polarity (+ve & -ve) within mass range of 50 to 800 amu. Cone voltage was optimized at 30V.

Preparation of diluent

90% v/v methanol in water was prepared and sonicated for 5 minutes.

Preparation of Standard solution for Related Substances

520 ppm of Montelukast sodium standard stock was prepared. 5 ml of that stock was diluted to 50 ml with diluent. 500 ppm of Levocetirizine HCl standard stock was prepared. 5 ml of that stock was diluted to 50 ml with diluent.

10 ml of montelukast sodium and 6 ml of levocetirizine HCl standards were mixed in a 25 ml volumetric flash and volume was made up with diluent.

Preparation of Sensitivity Standard solution for Related Substances

5 ml of standard solution for related substances was diluted to 50 ml with diluent.

Preparation of Placebo for related substances

3.5 g of placebo was transferred to 50 ml volumetric flask. 30 ml of diluent was added and sonicated for 20 minutes. Volume was made up with diluent and mixed.

Preparation of Sample Solution for related substances

25 tablets were weighed and transferred to 50 ml volumetric flask. 30 ml of diluent was added and sonicated for 20 minutes. Volume was made up with diluent and mixed.

Preparation of Spiked Sample for related substances

25 tablets were weighed and transferred to 50 ml volumetric flask. 30 ml of diluent was added and sonicated for 20 minutes. All known impurities were spiked with this sample solution. Volume was made up with diluent and mixed.

Validation study for related substances

Specificity: Interference of blank and placebo were checked at the retention of impurities. Forced degradation was performed to evaluate the interference of the impurities at the retention of Montelukast sodium and Levocetirizine HCl. In forced degradation study, acid & base degradation, hydrolysis, oxidation, thermal degradation, photolytic degradation (UV & LUX light) studies have been performed to estimate the percentage of impurities generated. For source of UV and LUX light, Newtronic photostability chamber (Mumbai, India) was utilised.

Accuracy: Accuracy was performed in triplicate at 50%, 100% and 150% level for all known impurities.

Linearity: Linearity solutions (a series of diluted solution of known concentrations) of both drugs and their impurities were injected into liquid chromatography system. Responses were plotted against concentration. From the curve, correlation co-efficient was determined as an indication of linearity.

Establishment of RRF and LOQ: From slopes of impurities and standards, RRF and LOQ values were determined. RRF values were calculated through division of slope of impurity by slope of standard whereas LOQs were calculated through STEYX.

Robustness: Under robustness study, increment and decrement of flow and column oven temperature were checked. During deliberate change in flow and temperature, relative retention times of all impurities were measured.

Instrument and chromatographic condition in U-HPLC for Assay

Agilent (Model: 1260 Infinity II & DAD) (U-HPLC) were employed connected to Empower 3.0 data integrator module. The separation was performed using an analytical column Kinetex, Phenyl-Hexyl (100°A, 150x4.6 mm, 5µm) maintained at 30ºC±2ºC. The wavelength was optimized at 230 nm. Flow rate was set at 1.2 ml/min with injection volume of 20 µl. 90% methanol in water was used as needle wash. Two different mobile phases were mixed in a proportionate way to run this gradient programme. One mobile phase consisted of water and trifluoroacetic acid in the ratio of 1000:1.5. Another mobile phase was mixture of acetonitrile and trifluoroacetic acid in the ratio of 1000:1.5. At the beginning, both mobile phases were mixed at the ratio of 60:40. At 15 minute, ratio became 50:50 and continued it up to 20 minutes. Ratio again changed to 10:90 at 22 minute and remained same up to 25 minute. At 26 minute, ratio was returned to its initial position *i.e.* 60:40 and hold up to 35 minutes. So, run time is 35 minutes.

Preparation of Standard solution for Assay

520 ppm of Montelukast sodium standard stock and 500 ppm of Levocetirizine HCl standard stock were prepared. 4 ml of Montelukast Sodium stock solution and 5 mL of Levocetirizine HCl stock solution were mixed in a 25 ml volumetric flask. Volume was made up with diluent and mixed. It was filtered through 0.45 µm PVDF syringe filter and first 3 ml of filtrate was discarded.

Preparation of Sample solution for Assay

10 tablets (equivalent to 40 mg of Montelukast sodium and 25 mg of Levocetirizine HCl) were weighed and transferred to a 200 ml volumetric flask. About 160 ml of diluent was added and sonicated until complete disintegration of tablets. Volumetric flask was cooled to room temperature, diluted to volume up to the mark with diluent and mixed well. Solution was centrifuged at 5000 rpm for 15 minutes. 4mL of the supernatant liquid was transferred into a 10 ml volumetric flask, diluted to volume up to the mark with diluent and mixed well. Solution was filtered through 0.45 µm PVDF syringe filter. First 3 ml of the filtrate was discarded.

Validation study for Assay

Specificity: Placebo interference, impurity interference and forced degradation were performed to evaluate the interference at the retention of Montelukast sodium and Levocetirizine HCl. Placebo equivalent to amount present in unit dose was prepared in duplicate. Impurity interference was performed through spiked and non-spiked in triplicate. Known impurities of Montelukast sodium (Sulphoxide, Cis-Isomer, Michael adduct, Methyl Ketone and Methyl Styrene) and Levocetrizine Hydrochloride (4-Chloro Benzhydryl Piperazine and Amide Impurity) were injected for this purpose. Forced Degradation study was carried out under several stress conditions (Acid, Alkali, Peroxide, water, heat, LUX light and UV light).

Accuracy: Accuracy was performed in triplicate at 25%, 50%, 75%, 100% and 125% level for both Montelukast sodium and Levocetirizine HCl.

Precision: System Precision was assessed through injection of standard solution according to developed method. Method Precision was performed on six unit doses by using product, prepared samples and injected into U-HPLC.

Linearity: Linearity of the method was evaluated for Montelukast having concentrations from 8.016 ppm to 100.198 ppm and for Levocetirizine having concentrations from 5.034 ppm to 62.931 ppm.

Solution Stability of Standard, Samples and Mobile Phase: Bench top stability of standard and samples were performed by preparing standard (single preparation) and samples (duplicate) through keeping them on bench top and in refrigerator. Both sample and standard were analysed initially and after 24 hours followed by determination of similarity factor between two conditions for these two drugs. Similarly, mobile phase stability was performed through keeping the mobile phase on bench top for 5 days. System suitability parameters were checked initially, after 3 days and after 5 days.

Filter Validation for assay:

Filter validation was performed by thorough comparison between unfiltered standard and filtered standard (0.45µ PVDF and 0.45µ Nylon), centrifuged sample and filtered sample (0.45µ PVDF and 0.45µ Nylon).

RESULT AND DISCUSSION:

Development of analytical method for Related Substances:

This optimized method fulfilled the criteria of system suitability. USP tailing factor for Montelukast sodium and Levocetirizine HCl were 1.1 for both. USP plate counts of these two peaks were 433429 and 65121 correspondingly. Area ratios of response between two injections were 1.00 in both cases. %Recovery of both drugs in sensitivity solution were 101% for Montelukast sodium and 99% for Levocetirizine HCl.

Validation study for related substances

Specificity: Figure 3 showed the overlaid chromatograms of blank, placebo and spiked sample. From this figure, it is clearly visible that there is no interference of blank and placebo at the retention time of impurities as well as both drugs..

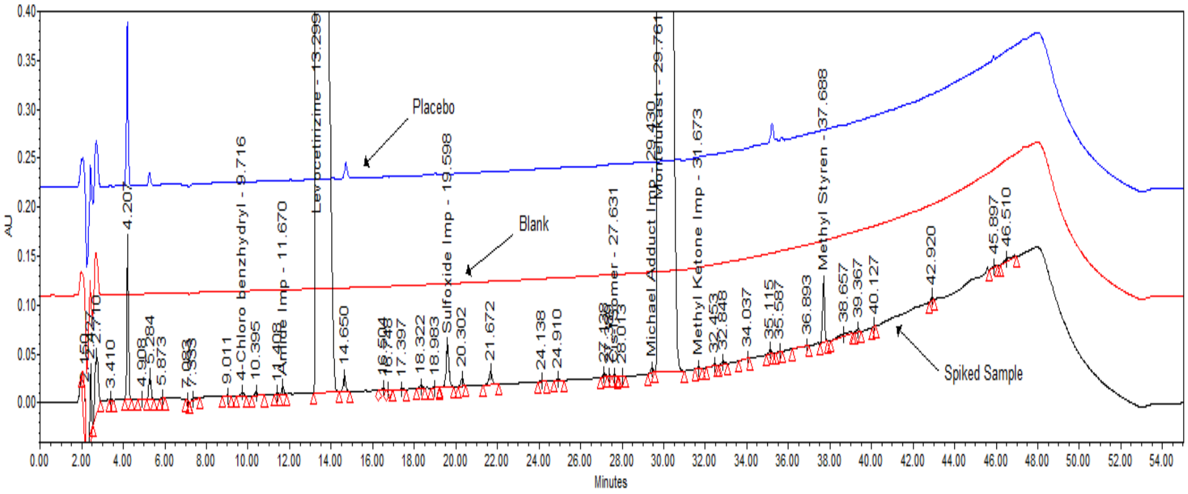


Fig. 3: Overlaid chromatograms of blank, placebo and spiked sample

In each stressed condition, percentage of impurity generated was calculated shown in Table 2 & Table 3. From these tables, it was observed that after degradation also, peaks were pure as purity threshold was greater than purity angle in all cases.

Table 2: Forced degradation study of Montelukast sodium

Condition	% Degradation	Purity angle	Purity Threshold
Acid Degradation (5mL of 1N HCl at 60°C for 30minutes)	4.7	0.061	1.003
Alkali Degradation (5mL of 1N NaOH at 60°C for 30 minutes)	0.4	0.064	1.003
Water Degradation (5mL at 60°C for 1 hour)	1.0	0.064	1.003
Peroxide Degradation (5mL of 3% H_2O_2 on Bench top for 10 minutes)	18.4	0.050	1.004
Heat Degradation (105°C for 72 hours)	7.8	0.057	1.005
UV Light Degradation (200 watt-hours/ Sq.meter)	14.6	0.066	1.012
LUX Light Degradation (1.2 Million Lux hours)	11.5	0.080	1.083

Table 3: Forced degradation study of Levocetirizine HCl

Condition	% Degradation	Purity angle	Purity Threshold
Acid Degradation (5mL of 1N HCl at 60°C for 30minutes)	1.4	0.693	1.045
Alkali Degradation (5mL of 1N NaOH at 60°C for 30 minutes)	0.4	0.307	1.051
Water Degradation (5mL at 60°C for 1 hour)	0.6	0.289	1.048
Peroxide Degradation (5mL of 3% H_2O_2 on Bench top for 10 minutes)	1.7	0.616	1.167
Heat Degradation (105°C for 72 hours)	3.3	0.629	1.069
UV Light Degradation (200 watt-hours/ Sq.meter)	0.6	0.634	1.168
LUX Light Degradation (1.2 Million Lux hours)	0.5	0.746	1.608

Accuracy: % recoveries of all known impurities were represented in Table 4. The results proved that all values were within limit.

Table 4: Accuracy study of different impurities

% Level	% Recovery						
	Sulfoxide	Cis-isomer	Michael adduct	Methyl ketone	Methyl styrene	Amide	4-Chloro Benzhydryl Piperizine
50	98.4	99.1	98.6	97.6	98.1	99.1	99.3
	98.8	99.5	98.7	97.9	98.5	97.9	99.8
	97.7	98.8	97.8	97.1	99.0	99.4	98.7
100	99.9	100.2	99.2	99.1	99.9	100.2	99.6
	99.7	99.7	99.5	98.8	101.2	100.8	99.1
	100.2	101.1	99.4	99.5	100.3	101.1	100.3
150	100.5	101.3	100.1	100.9	100.5	101.3	100.9
	100.8	101.5	100.6	100.4	100.9	101.7	101.2
	100.9	100.9	100.7	100.3	101.2	100.6	100.7

Linearity:

Linearity of both drugs and their related substances were represented in Figure 4 to Figure 12. All correlation co-efficient were above 0.99.

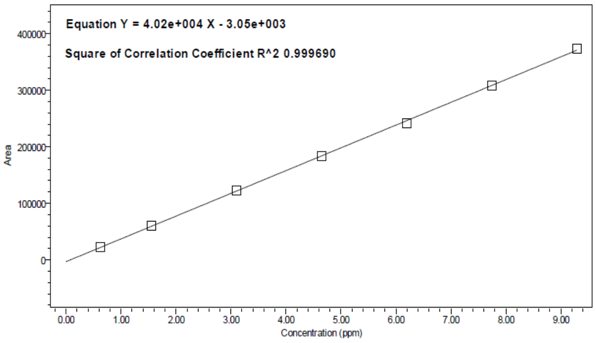


Fig. 4: Linearity curve of Montelukast

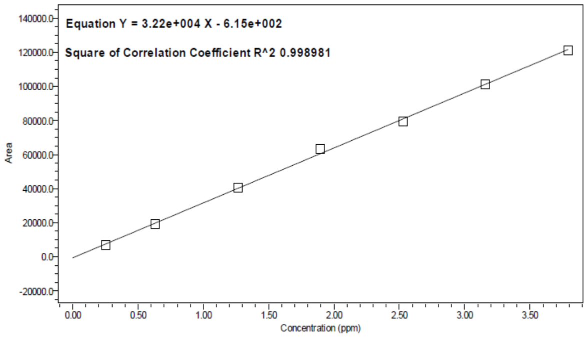


Fig. 5: Linearity curve of Levocetirizine

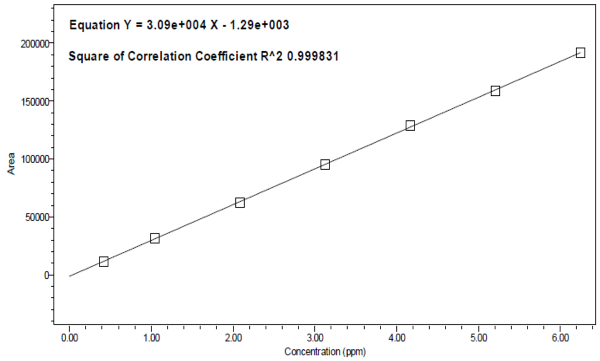


Fig. 6: Linearity curve of Montelukast Sulfoxide

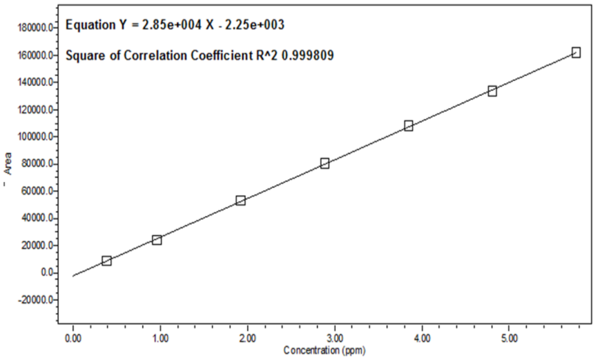


Fig. 7: Linearity curve of Michael adduct

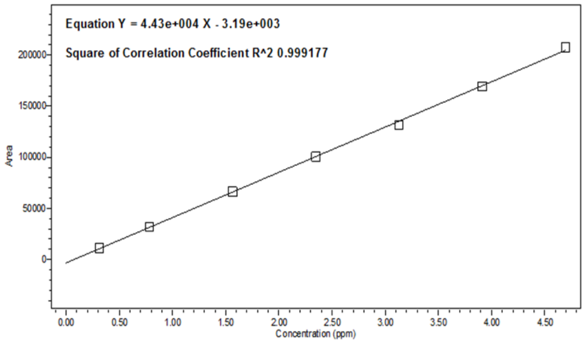


Fig. 8: Linearity curve of Cis-isomer

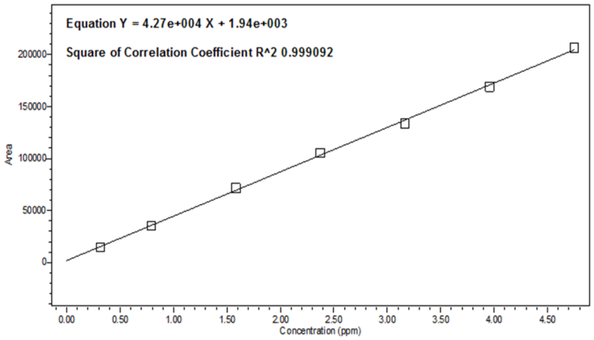


Fig. 9: Linearity curve of Montelukast methyl ketone

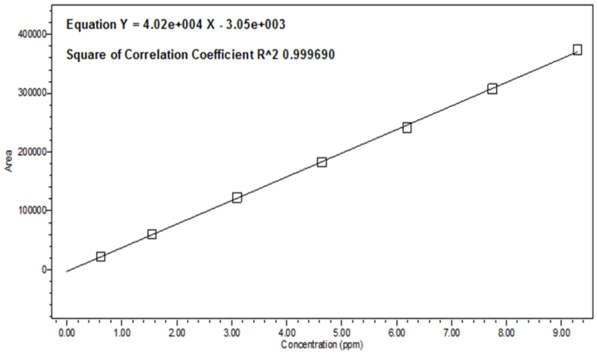


Fig. 10: Linearity curve of Methyl styrene

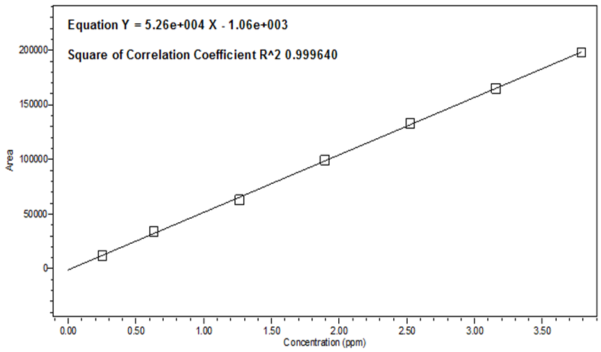


Fig. 11: Linearity curve of 4-Chloro Benzhydryl Piperizine

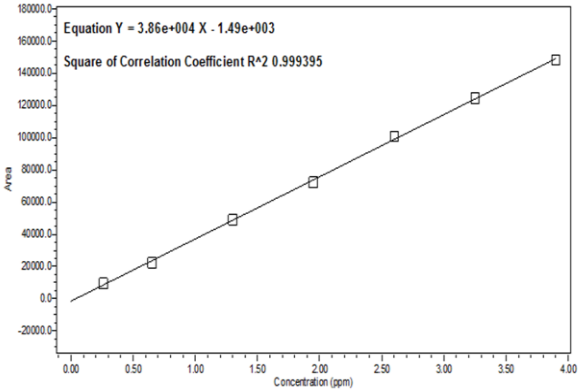


Fig. 12: Linearity curve of Amide impurity

Establishment of RRF and LOQ: RRF values of Montelukast impurities i.e. sulfoxide, cis-isomer, Michael adduct, methyl ketone, methyl styrene were 0.86, 1.31, 1.24, 0.80, 1.19, 1.12 respectively whereas impurities of Levocetirizine i.e. 4-chlorobenzhydryl piperizine, amide were 1.63, 1.20 respectively. Limit of quantification for Montelukast sodium, Sulphoxide, Cis-isomer, Methyl ketone, Michael adduct, Methyl styrene, Levocetirizine HCl, Amide, 4-Chloro benzhydryl piperazine were 0.9374 ppm, 0.3067 ppm, 0.7011 ppm, 0.5092 ppm, 0.541 ppm, 0.3015 ppm, 0.6187 ppm, 0.4580 ppm, 0.3633 ppm, 0.2716 ppm respectively. In Figure 13, both drugs and their impurities were represented at LOQ level.

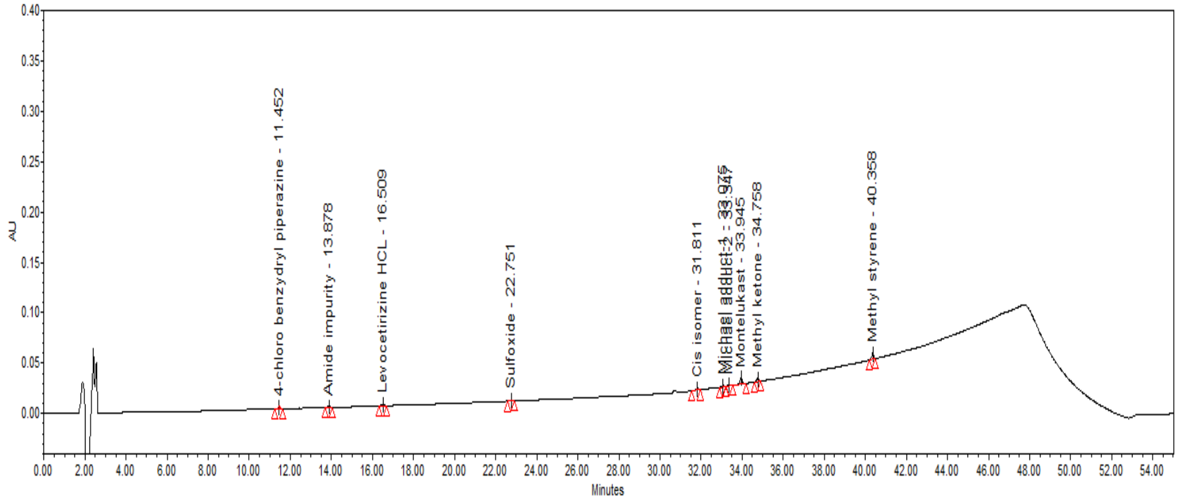


Fig. 13: All impurities with both drugs at LOQ level

Robustness

Relative retention times of all impurities were shown in Table 5. From that table, it was observed that there was no significant difference in relative retention time of all impurities proving the robustness of the method.

Table 5: Robustness study with respect to flow and column oven temperature

	Decrement of Flow		Increment of Flow		Decrement of Temp		Increment of Temp	
Name of Impurity	RT	RRT	RT	RRT	RT	RRT	RT	RRT
Sulfoxide Imp	23.432	0.69	21.813	0.67	22.033	0.67	21.568	0.67
Cis-Isomer	32.402	0.95	30.699	0.95	30.994	0.95	30.338	0.95
Michael Adduct	33.631	0.99	32.141	0.99	32.408	0.99	31.817	0.99
Methyl Ketone	35.386	1.04	33.942	1.05	34.322	1.05	33.519	1.05
Methyl Styrene	40.868	1.2	39.544	1.22	39.828	1.22	39.22	1.22
4-Chloro benzahydral Piperazine	11.969	0.74	10.59	0.73	10.551	0.71	10.551	0.74
Amide	14.271	0.88	12.799	0.88	13.028	0.87	12.537	0.88

LC-MS:

Mass spectra of Montelukast and its impurities were shown in Figure 14 to Figure 19. Figure 20 to Figure 22 represented the mass spectra of Levocetirizine HCL and its impurities. All mass values were distinguished and determined suitably.

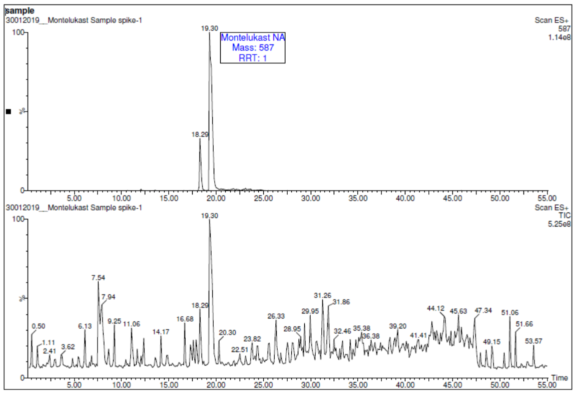


Fig. 14: Mass spectra of Montelukast

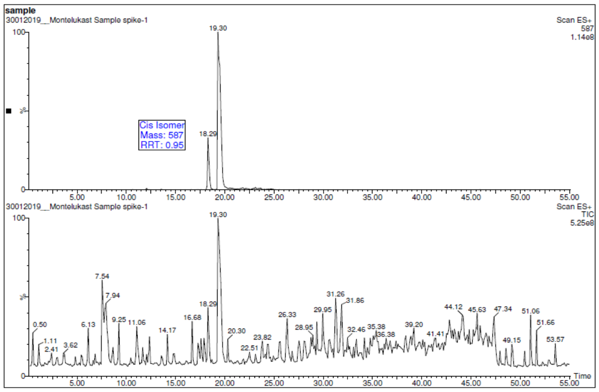
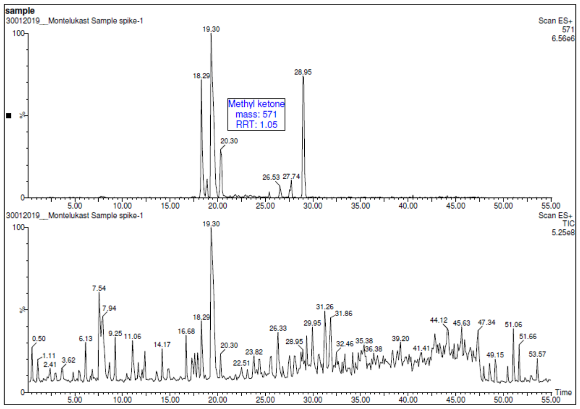
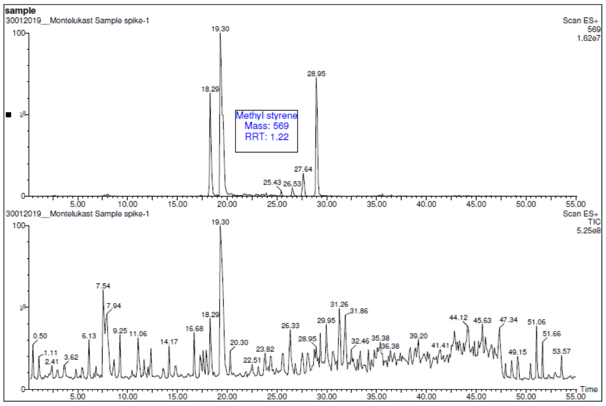


Fig. 15: Mass spectra of Cis-isomer



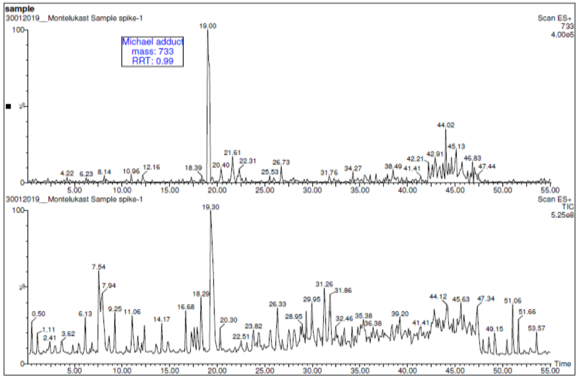
Methyl ketone
mass: 571
RRT: 1.05

Fig. 16: Mass spectra of Methyl ketone



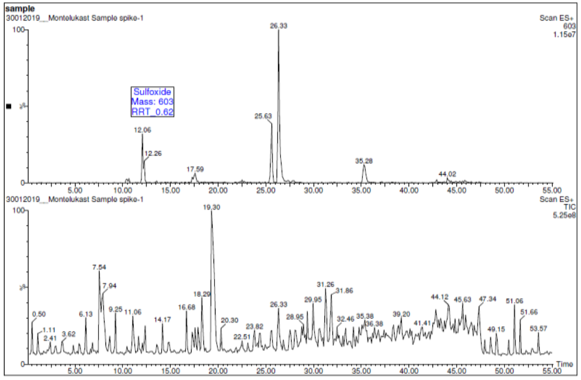
Methyl styrene
Mass: 569
RRT: 1.22

Fig. 17: Mass spectra of Methyl styrene



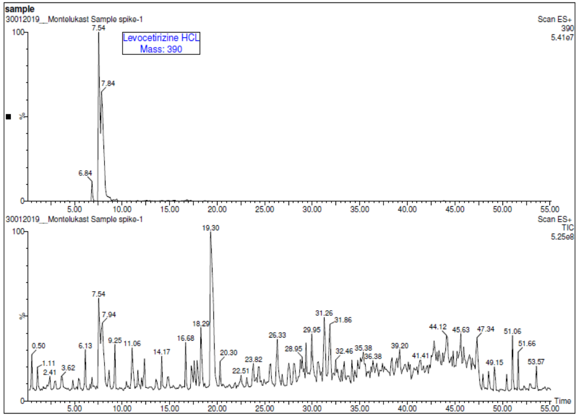
Michael adduct
mass: 733
RRT: 0.99

Fig. 18: Mass spectra of Michael adduct



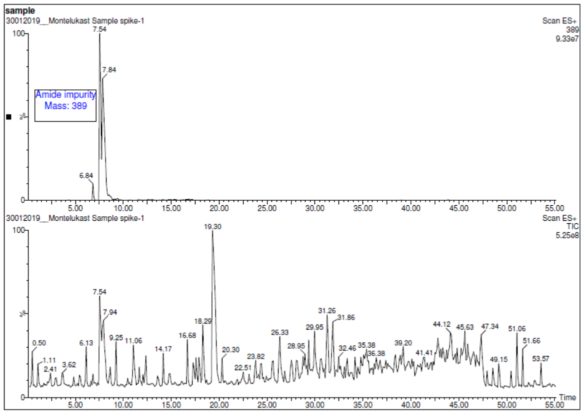
Sulfoxide
Mass: 603
RRT_0.62

Fig. 19: Mass spectra of Sulfoxide



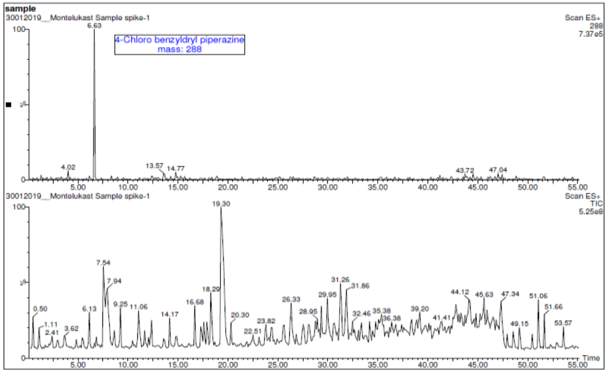
Levocetirizine HCL
Mass: 390

Fig. 20: Mass spectra of Levocetirizine HCl



Amide impurity
Mass: 389

Fig. 21: Mass spectra of Amide impurity



4-Chloro benzyldryl piperazine
mass: 288

Fig.22: Mass spectra of 4-Chloro benzyldryl piperizine

Development of analytical method for Assay:
This optimized method fulfilled the criteria of system suitability. USP tailing factor for Montelukast sodium and Levocetirizine HCl were 1.4 and 0.9 respectively. USP plate of these peaks were 51490 and 11513 correspondingly. %RSD of five replicates of Montelukast sodium and Levocetirizine HCl were 0.2% and 0.4% respectively.

Validation:
Specificity: Overlaid chromatograms of blank, placebo and standard were shown in Figure 23. It was clearly visible that there was no interference at the retention time of Montelukast sodium and Levocetirizine HCl from placebo.

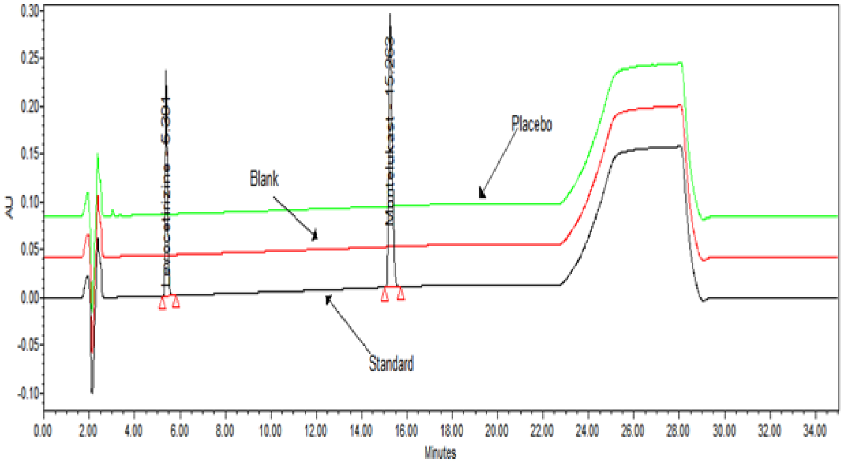


Fig. 23: Overlaid chromatograms of Blank, Placebo and Standard

Assay was determined for non-spiked sample and sample spiked with known impurities. But there was no significant difference in assay value between spiked and non-spiked shown in Table 6. It was also observed that no known impurity showed any interference at retention times of analytic peaks observed in the Table 7 and Figure 24.

Table 6: Assay Difference between non-spiked and spiked samples

Name of Sample	Levocetirizine		Montelukast	
	% Assay in non-spiked sample	% Assay from spiked Sample	% Assay in non-spiked sample	% Assay from spiked Sample
Test Preparation-1	101.4	102.4	106.2	107.7
Test Preparation-2	101.3	102.3	106.2	107.5
Test Preparation-3	101.1	102.4	106.1	107.6
Average	101.3	102.4	106.2	107.6
% Difference	1.1		1.4	

Table 7: Impurity Interference

Name of Impurity	RT from Individual Injection (min)	RT from Impurity Blend (min)	RT from Spiked Sample (min)
Levocetirizine Impurities			
4-Chloro Benzhydryl Piperazine	4.101	4.098	4.084
Amide Impurity	4.457	4.448	4.428
Montelukast impurities			
Sulfoxide	7.577	7.523	7.470
Cis-Isomer	12.955	12.900	12.820
Michael adduct-1	13.500	13.479	13.387
Michael adduct-2	13.766	13.717	13.623
Methyl Ketone	16.259	16.167	16.069
Methyl Styrene	23.855	23.844	23.804

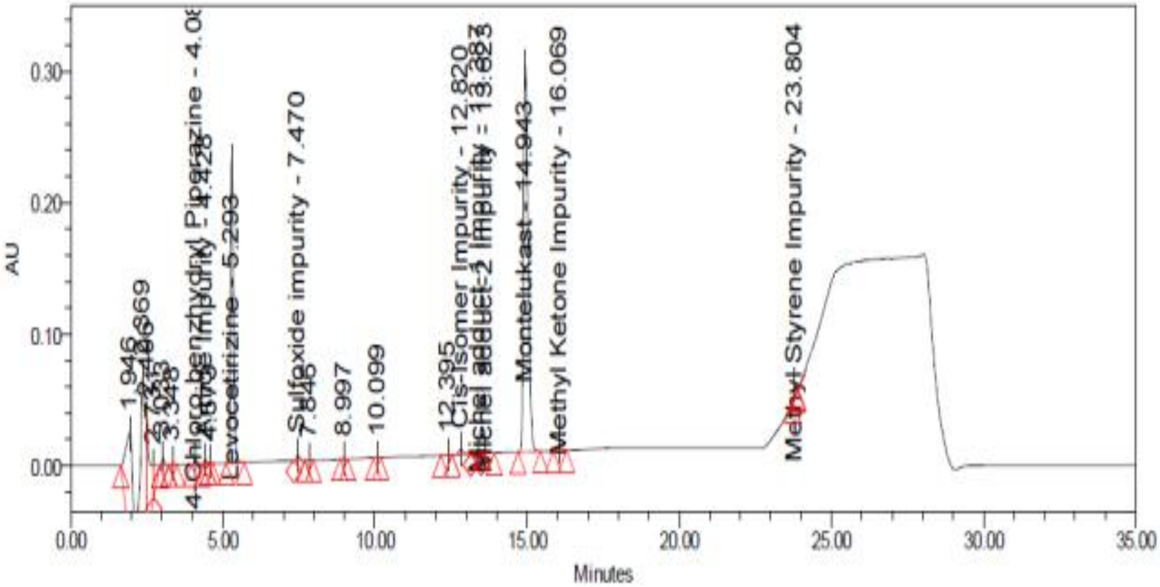


Fig. 24: Chromatogram of Spiked Sample

Accuracy: From Table 8 and Table 9, it was clear that all recovery values were within limit and close to 100%.

Table 8: Accuracy for Levocetirizine

% Level	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery
25	6.26	6.41	102.4
	6.26	6.36	101.6
	6.26	6.35	101.4
50	12.53	12.59	100.5
	12.53	12.70	101.4
	12.53	12.74	101.7
75	18.86	18.96	100.5
	18.91	18.79	99.4
	18.87	18.85	99.9
100	25.26	25.00	99.0
	25.26	24.97	98.9
	25.21	24.99	99.1
125	31.69	31.15	98.3
	31.56	31.15	98.7
	31.57	31.11	98.5

Table 9: Accuracy for Montelukast

% Level	Amount Added (ppm)	Amount Recovered (ppm)	% Recovery
25	10.17	10.43	102.6
	10.17	10.32	101.5
	10.17	10.31	101.4
50	20.35	20.48	100.6
	20.35	20.62	101.3
	20.35	20.71	101.8
75	30.14	30.68	101.8
	30.11	30.48	101.2
	30.08	30.55	101.6
100	40.06	40.56	101.2
	40.10	40.50	101.0
	40.21	40.52	100.8
125	50.06	50.60	101.1
	49.99	50.60	101.2
	50.10	50.55	100.9

Precision: %RSD of six injections of Montelukast sodium and Levocetirizine HCl standards were 0.3 and 0.6 respectively whereas method precisions for these standards were 0.6% for both cases.

Linearity: From the results of linearity solutions of Montelukast sodium and Levocetirizine HCl (10%, 25%, 50%, 75%, 100% and 125% of target concentrations (80 ppm for Montelukast and 50 ppm for Levocetirizine)), the co-relation coefficient of these standards were 1.000 shown in Figure 25 and Figure 26.

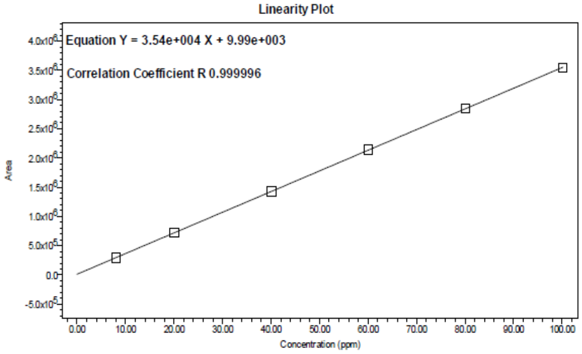


Fig. 25: Linearity graph for Montelukast

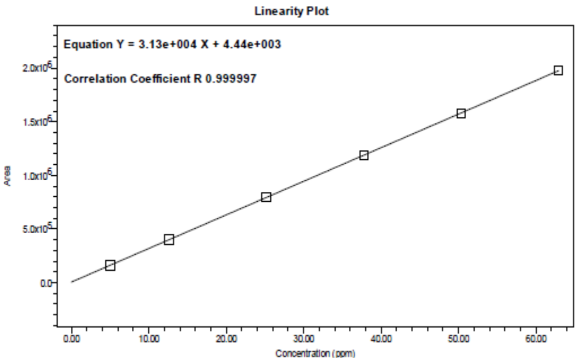


Fig. 26: Linearity graph for Levocetirizine

Solution stability of standard, sample and mobile phase: Similarity factors of both standards injected after 28 hours with respect to initial were tabulated in Table 10. The results proved that standard solution is stable. Assay was determined for initial sample and sample injected after 26 hours. There was no substantial variation observed in Table 11. System suitability parameters were shown in Table 12 after using same mobile phase at interval (3 days and 5 days). All system suitability parameters were within limit.

Table 10: Stability of Standard solution

Interval	Similarity factor	
	Levocetirizine	Montelukast
Initial	NA	NA
28 hours	1.00	1.01

Table 11: Stability of Sample solution

For Levocetirizine:

Interval	**% Assay**		**% Difference**	
	Sample-1	**Sample-2**	**Sample-1**	**Sample-2**
Initial	100.6	100.9	NA	NA
26 hours	100.3	100.6	0.3	0.3

For Montelukast:

Interval	**% Assay**		**% Difference**	
	Sample-1	**Sample-2**	**Sample-1**	**Sample-2**
Initial	106.7	107.5	NA	NA
26 hours	107.8	108.7	1.1	1.2

Table 12: Mobile Phase Stability

S No	**Parameter**	**Result**			**Acceptance Criteria**
		Initial	**After 3 days**	**After 5 Days**	
1	USP Tailing factor for Levocetirizine peak	0.9	1.2	1.2	NMT 2.0
2	USP Tailing factor for Montelukast peak	1.4	1.3	1.3	NMT 2.0
3	Theoretical Plate count for Levocetirizine peak	11513	12947	12890	NLT 2000
4	Theoretical Plate count for Montelukast peak	51490	46905	47438	NLT 2000
5	% RSD for peak areas of Levocetirizine from five replicate injections of standard	0.4	0.1	0.1	NMT 2.0
6	% RSD for peak areas of Montelukast from five replicate injections of standard	0.2	0.2	0.0	NMT 2.0

Filter validation for assay:
Similarity factor was checked to perform filter validation. Results of similarity factors were tabulated in Table 13 and Table 14. The values proved that there was no such variation among unfiltered, filtered through PVDF and filtered through Nylon.

Table 13: Filter Variability for Standard

Parameter	**Similarity Factor**		**Acceptance Criteria**
Similarity factor between two unfiltered Standard Solutions	**Levocetirizine**	**Montelukast**	
	0.99	0.99	Should be in between 0.98 to 1.02

Name of Analyte	**Similarity Factor**			
	Standard-1		**Standard-2**	
	0.45µ PVDF	**0.45µ Nylon**	**0.45µ PVDF**	**0.45µ Nylon**
Levocetirizine	1.00	1.00	1.00	1.00
Montelukast	1.00	1.00	1.00	1.01

Table 14: Filter Variability for Sample

Name of Analyte	Similarity Factor			
	Sample-1		Sample-2	
	0.45µ PVDF	0.45µ Nylon	0.45µ PVDF	0.45µ Nylon
Levocetirizine	1.00	1.00	1.00	1.00
Montelukast	1.00	0.99	1.00	0.99

CONCLUSION:

This gradient reverse-phase U-HPLC method developed for quantification of related substances in combined dosage form of Montelukast sodium and Levocetirizine HCl was specific, accurate, linear, precise and robust. This analytical method was validated and showing satisfactory data for all the validation parameters tested. In related substance method, all impurities were well separated along with establishment of their limit of quantification values. From the LC-MS data, it was clear that this related substance method can also be applied for mass determination of these drugs with their impurities. It proves that related substance method is mass compatible. Assay method developed for simultaneous determination of both drugs was specific, accurate, linear, precise and robust.

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