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A RAPID AND SENSITIVE METHOD FOR THE QUANTITATION OF MONTELUKAST IN HUMAN PLASMA USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

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ABSTRACT

A simple, precise and reproducible liquid chromatography–tandem mass spectrometry method has been developed and validated according to the United States - Food and Drug Administration (US-FDA) guidelines for the quantitation of Montelukat in human plasma using Neverapine as an internal standard. Montelukast is a fast acting and potent cysteinyl leukotriene receptor antagonist which is being used in the treatment of asthma. Quantitation was performed on a triple quadrupole mass spectrometer employing electrospray ionization technique, operating in multiple reaction monitoring and positive ion mode. The chromatographic run time was 3.2 mins on a Agilent Zorbax SB C8, 150x4.6 mm, 5.0µm Column. The mobile phase was a mixture of Methanol: 0.1 % Formic acid in water (90:10 v/v). Inter-batch and intra-batch coefficient of variation across four validation runs for the quality control samples was less than 6.02%. The accuracy determined at quality control levels was within 90.98 – 111.07%.

INTRODUCTION: Montelukast sodium [1- {[(1 (R)- (3- (2- (7-chloro- 2- quinolinyl)- (E)- ethenyl) phenyl)-3-(2-(1-hydroxy-1-methylethyl) phenyl) propyl) thio] methyl} cyclopropylacetic acid sodium salt] is a potent and selective antagonist of the cysteinyl leukotriene (Cys-LT1) receptor and is used for the treatment of bronchial asthma and seasonal allergic rhinitis [1]. Montelukast can be administered orally once daily thereby increasing compliance over other common asthma treatments, has no known adverse effects or drug interactions, has demonstrated efficacy against allergen or exercise-induced bronchoconstriction (EIB) and is the only leukotriene modifier approved by the US Food and Drug Administration for use by children [2, 3] from 2 to 12 years of age. Leukotriene receptor antagonists block the effects of cysteinyl Leukotrienes in the air ways. This results in a reduction in bronchoconstriction, mucous secretion, vascular permeability and eosinophil recruitment. It also inhibits both early and late stage bronchoconstriction, implying both an anti-inflammatory and bronchodilatory action [4]. Montelukast prevents tumor cell migration through both peripheral and cerebral capillaries [5]. It is an effective monotherapy controller in children with mild asthma [6]. Topical ocular montelukast can be a potential therapeutic drug with a new route of administration that can be used for treatment of allergic conjunctivitis [7].

In the present study, an LC -MS-MS method has been developed and validated for the quantitation of Montelukast in human plasma using Nevirapine as internal standard. The method was validated for selectivity, sensitivity, recovery, linearity, accuracy and precision, and stability studies according to the US-FDA guidelines.

EXPERIMENTAL DESIGN:

Chemicals and Reagents: Montelukast obtained from Unimark Remedies, Mumbai, India. Methanol (HPLC Grade) Glacial Acetic Acid (HPLC Grade) Formic Acid (GR Grade) Liquor Ammonia (SQ Grade) MilliQ Water (HPLC Grade) Phenomenex strata X 30mg/1mL cartridges were used. All aqueous solutions including the buffer for the mobile phase were prepared with water (resistivity of 18.2 MΩ cm) collected from a Milli-Q gradient system of Millipore (Elix 3, Milli-Q A10 Academic). The blank human plasma with EDTA–K_2 anticoagulant was collected from Clinical Pharmacological Unit of Azidus Laboratories, Chennai, India.

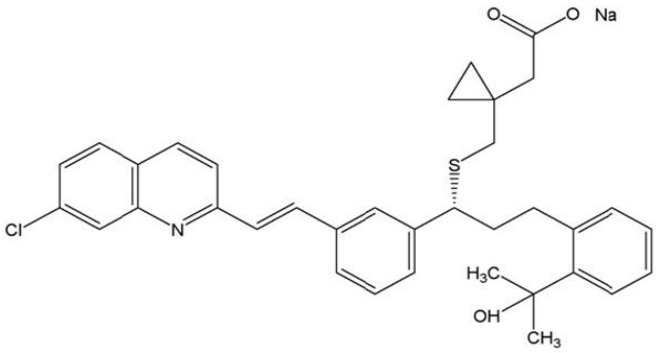

FIGURE 1: CHEMICAL STRUCTURE OF MONTELUKAST SODIUM

Instrumentation and Chromatographic conditions: Analysis was performed on a Agilent 6460 Triple Quad LC/MS, Agilent Infinity 1290-Binary Pump, Auto Sampler, Degasser. Quantitation was achieved by MS – MS detection in positive ion mode as ESI and Agilent jet stream as ion source. The mass spectrometer conditions are, Gas temperature 300°C, gas flow 8 L/Min, Nebulizer 45 Psi, Sheath gas temperature 350°C, Sheath gas flow 11 L/Min, Capillary 4000 V and Nozzle 500 V. MRM parameters Montelukast precursor ion at the *m/z* 586, product ion at *m/z* 422 and fragmentor ion at *m/z* 140. Collision energy is 21V.

Chromatographic conditions were optimized by using Agilent Zorbax SB C8, 150x4.6 mm, 5.0µm, Mobile phase is Methanol: 0.1 % Formic acid in water (90:10 v/v), Flow rate 1.0mL/Min, Auto sampler temperature 4°C, Injection Volume 10 µL and run time is 3.2 Mins.

Standard Solutions: Montelukast sodium was weighed equivalent to about 10mg of Montelukast and transfer into a 10 mL volumetric flask. Dissolve it in Methanol and make up the volume with the same to produce a solution of 1 mg/mL strength of Montelukast.

Preparation of Nevirapine (IS) Stock Solution: Nevirapine was weighed equivalent to about 10 mg of Nevirapine and transfer into a 10 mL volumetric flask. Dissolve it in Methanol and make up the volume with the same to produce a solution of 1 mg / mL strength of Nevirapine.

Preparation of Calibration and Quality Control Samples: From Montelukast stock solution, Stock Dilutions ranging from 232.5361 ng/mL- 32404.6958 ng/mL with (Methanol: Water / 1:1) were prepared. Then calibration standards in Human K_2ETDA plasma ranging from 4.6507ng/mL- 648.0939 ng/mL were prepared (**Table 1**).

Preparation of Montelukast QC Samples Dilutions: From Montelukast Stock Solution, Stock Dilutions ranging from 240.4182ng /mL- 25288.6941 ng /mL with (Methanol: Water / 1:1) were prepared. Then Quality Control samples in human K_2ETDA plasma ranging from 4.8084ng/mL- 505.7739 ng/mL, were prepared (**Table 2**).

Preparation of Nevirapine (IS) Stock Dilutions: From Nevirapine Stock Solution, Working Concentration of the Internal Standard Solution (10000.000 ng/mL) was prepared (**Table 3**).

TABLE - 1: MONTELUKAST CALIBRATION STANDARDS IN HUMAN PLASMA

INITIAL SOLUTION LD	INITIAL STOCK CONC. (ng/ml)	VOLUME OF STOCK TAKEN (mL)	VOLUME OF PLASMA ADDED (mL)	TOTAL VOLUME OF FINAL SOLUTION (mL)	FINAL STOCK CONC. (ng/mL)	FINAL SOLUTION LD
SS1	32404.6958	0.200	9.800	10.000	648.0939	STD-8
SS2	25923.7567	0.200	9.800	10.000	518.4751	STD-7
SS3	16850.4418	0.200	9.800	10.000	337.0088	STD-6
SS4	10110.2651	0.200	9.800	10.000	202.2053	STD-5
SS5	4044.1060	0.200	9.800	10.000	80.8821	STD-4
SS6	2022.0530	0.200	9.800	10.000	40.4411	STD-3
SS7	465.0722	0.200	9.800	10.000	9.3014	STD-2
SS8	232.5361	0.200	9.800	10.000	4.6507	STD-1

TABLE- 2: MONTELUKAST QUALITY CONTROL SAMPLES IN HUMAN PLASMA

INITIAL SOLUTION LD	INITIAL STOCK CONC. (ng/ml)	VOLUME OF STOCK TAKEN (mL)	VOLUME OF PLASMA ADDED (mL)	TOTAL VOLUME OF FINAL SOLUTION (mL)	FINAL STOCK CONC. (ng/mL)	FINAL SOLUTION LD
QC1	25288.6941	0.200	9.800	10.000	505.7739	HQC
QC2	12998.3887	0.200	9.800	10.000	259.9678	MQC
QC4	4159.4844	0.034	9.966	10.000	14.1422	LQC
QC5	707.1123	0.068	9.932	10.000	4.8084	LOQQC

TABLE 3: STOCK DILUTION FOR NEVIRAPINE

INITIAL SOLUTION LD	INITIAL STOCK CONC. (ng/ml)	VOLUME OF STOCK TAKEN (mL)	VOLUME OF PLASMA ADDED (mL)	TOTAL VOLUME OF FINAL SOLUTION (mL)	FINAL STOCK CONC. (ng/mL)	FINAL SOLUTION LD
STOCK A	1000000.000	0.100	9.900	10.000	10000.000	STOCK B

Sample Preparation: Routine daily calibration curves and quality controls were thawed at room temperature. Exactly 300µL of spiked plasma samples were transferred to the appropriately labeled tubes. Then 50µL of internal standard (Nevirapine, 10000.000 ng/mL in Methanol: Water/1:1/(v/v) was added to all the tubes except for tubes labeled as Blank. After vortexing 250µL of 10 % Acetic acid in water was added. Phenomenex strata X 30mg/1mL cartridges were conditioned with 1mL methanol followed by equilibration with 1 mL 10 % Acetic acid in water. Sample was loaded and washed with 1 mL 10% Methanol in water, then eluted with 0.5 mL x 2 times with 0.5 % Ammonia in methanol. After eluting the samples were evaporated at 40º C for about 15 minute s by using Low Volume evaporator. The samples were reconstituted with mobile phase (Methanol: 0.1% Formic acid in water / 90:10 (v/v). Reconstituted samples were vortexed for 1 min, and then the samples were transferred into 350µL inserts in appropriately labeled auto sampler vials.

Method Validation: The method was validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation [8, 9].

Specificity Selectivity: The specificity of the method was determined by analyzing six different batches of human plasma with one lipemic and one hemolyzed samples to evaluate the specificity of the method, along with Aqueous Solution equivalent to LLOQ concentration with intended IS Concentration was injected. This is to demonstrate the lack of chromatographic interference from endogenous plasma components.

Recovery: The recovery of Montelukast and IS was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 4) with the response of analytes from post extracted plasma standard sample at equivalent concentrations [8]. Recoveries was determined at low, mid and high quality control concentrations, whereas the recovery of the IS was determined at a single concentration of 10000.000 ng/mL.

Matrix Effect: The effect of plasma constituents over the ionization of analytes and IS was determined by comparing the aqueous solution containing analyte equivalent to LQC concentration and six processed matrix samples including one lipemic and one hemolyzed with post spiked analyte at LQC level and intended IS concentration [8-9].

Calibration Curve: Calibration curves were acquired by plotting the peak-area ratio of the transition pair of analytes to that of IS against the nominal concentration of calibration standards. The analyte concentration of the different CC and QC samples are shown in Table 1 and 2. The results were fitted to linear regression analysis. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set at ±20% [8, 9].

Precision and Accuracy: The method has been found to be reproducible by performing three Precision and Accuracy (P&A) batches consisting of intraday batch and inter day batches. Each analytical run in P&A consists of Standards at LLOQ and ULOQ of one replicate at other levels along with 6 replicates of QC at all levels. The criteria for acceptability of the data included accuracy within ±15% deviation (SD) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of SD [8, 9]. Intraday run is evaluated from the Precision and Accuracy of 6 replicates of QC samples at LOQQC, LQC, MQC and HQC levels from the first three accepted analytical runs individually. Inter day run is evaluated from the Precision and Accuracy of 18 replicates of QC samples at LOQQC, LQC, MQC and HQC levels obtained from the first three accepted analytical runs.

Stability Experiments: The stability of analytes and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 24 h (in auto-sampler at 4 ^{o}C) after the initial injection. The peak-areas of the analytes and IS obtained at initial cycle were used as the reference to determine the relative stability of the analytes at subsequent points. Stability of analytes in the biomatrix after 8 h exposure in bench top was determined at two concentrations in six replicates. Freezer stability of the analytes in biomatrix was assessed by analyzing the QC samples stored at 20^{o}C for at least 30 days. The stability of analytes in biomatrix following repeated three freeze-thaw cycles (stored at –20 C between cycles) was assessed using QC samples spiked with analytes. Samples were processed as described under as above. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e., ±15% SD) and precision (i.e., ±15% RSD) [8, 9].

RESULTS:

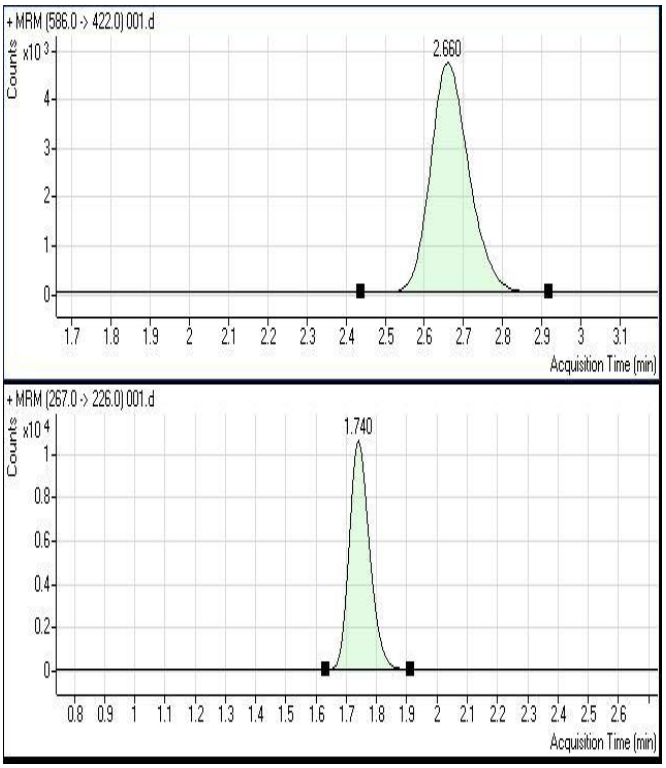

FIGURE 2: REPRESENTATIVE CHROMATOGRAM OF AN AQUEOUS SAMPLE

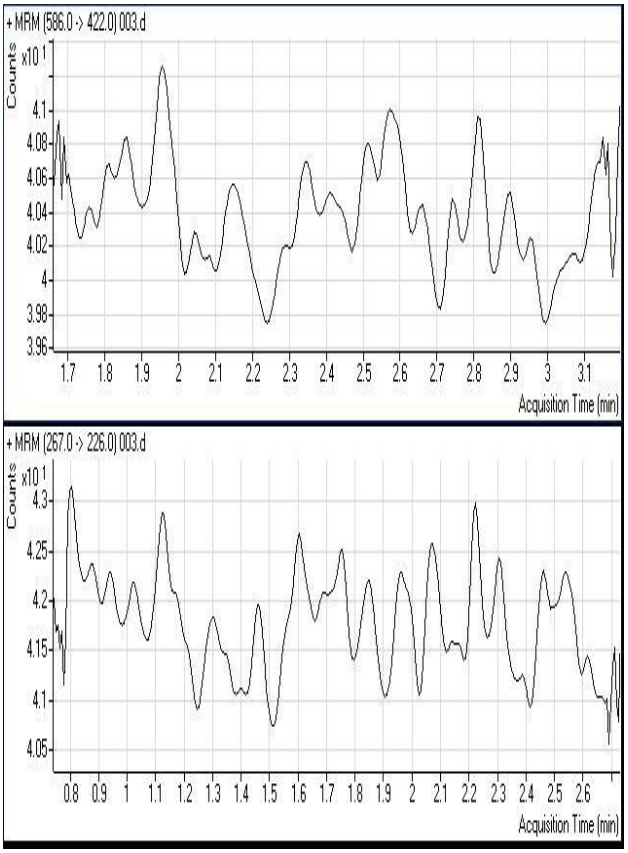

FIGURE 3: REPRESENTATIVE CHROMATOGRAM OF A BLANK MATRIX SAMPLE

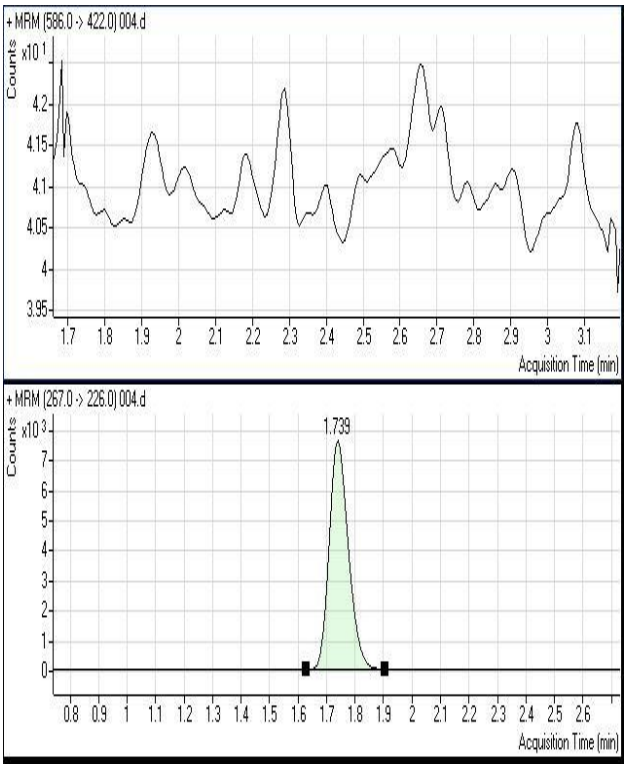

FIGURE 4: REPRESENTATIVE CHROMATOGRAM OF A BLANK MATRIX SAMPLE WITH INTERNAL STANDARD

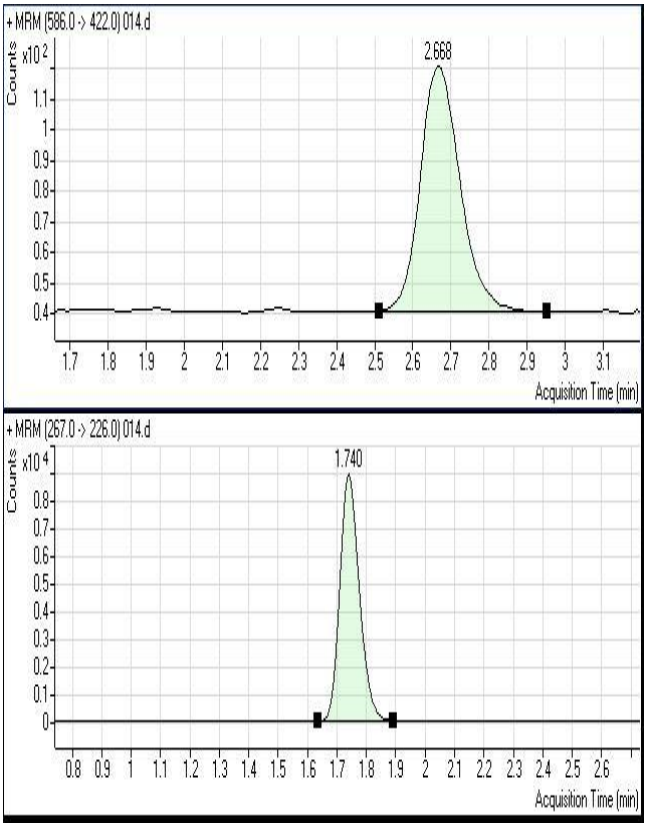

FIGURE 5: REPRESENTATIVE CHROMATOGRAM OF LOQQC SAMPLE

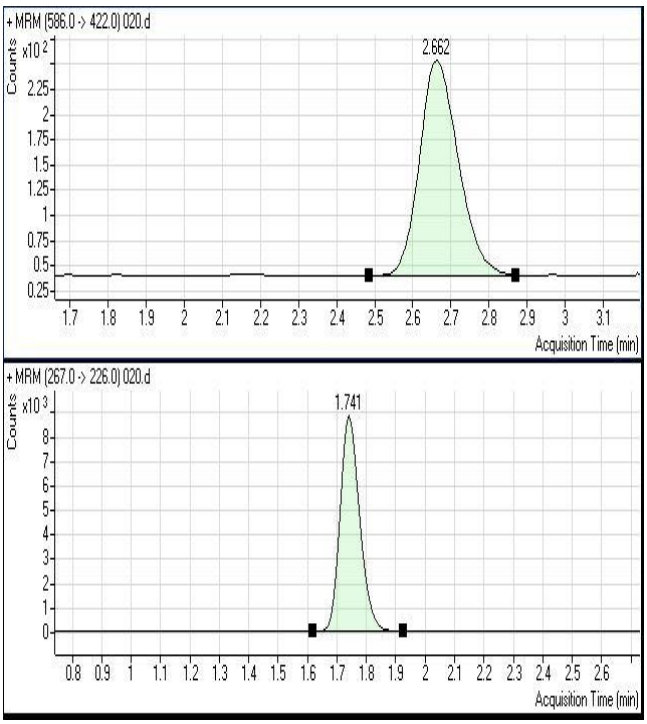

FIGURE 6: REPRESENTATIVE CHROMATOGRAM OF LQC SAMPLE

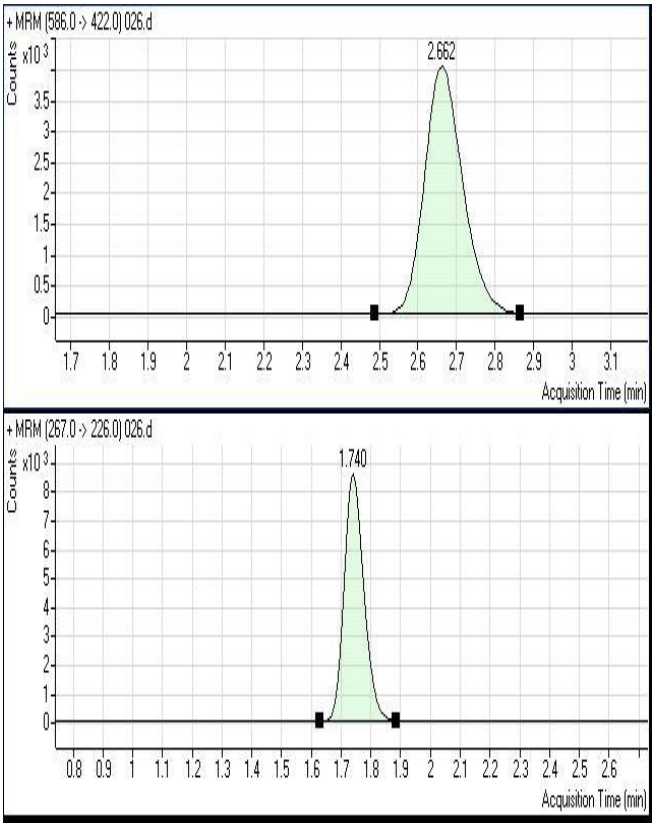

FIGURE 7: REPRESENTATIVE CHROMATOGRAM OF MQC SAMPLE

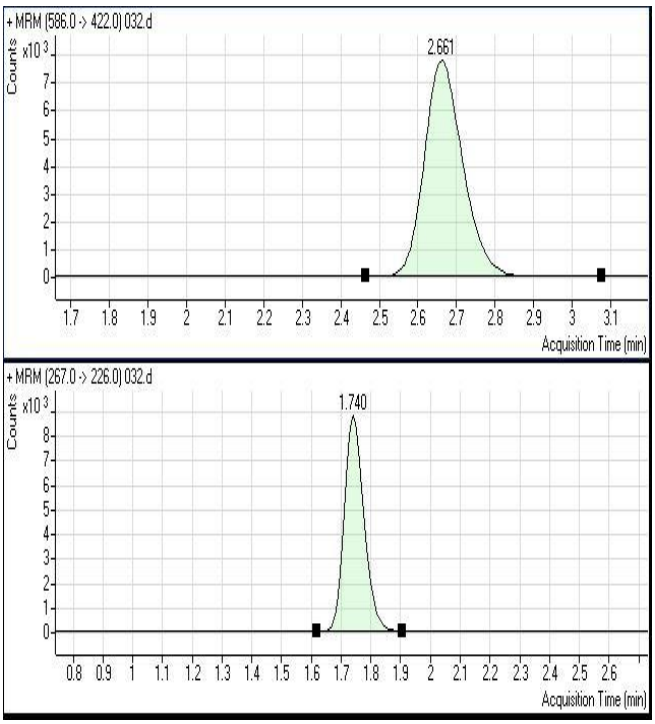

FIGURE 8: REPRESENTATIVE CHROMATOGRAM OF HQC SAMPLE

Specificity Selectivity: The percentage of interfering Peaks in the retention time of analyte for the six lots was 0.00, 0.00, 0.00, 0.00, 0.00 and 8.28. The percentage of interfering Peaks in the retention time of IS for the six lots were 0.00, 0.00, 0.00, 0.00, 0.00 and 0.01. Percentage CV of the six replicates of aqueous LLOQ for Analyte was 2.68.

Recovery: % CV for recovery of unextracted LQC, MQC, HQC and IS were 0.98%, 0.74%, 0.59% and 0.82% respectively. Percentage CV for recovery of extracted LQC, MQC, HQC and IS were 3.42%, 5.36%, 4.36% and 1.90% respectively. % CV of mean recovery of low, medium and high was 0.81% Mean percentage of Analyte Recovery was 56.08% and IS was 86.22%.

Matrix Effect: The Percentage of Matrix Effect for each Lot of Analyte was -5.86,-0.69,-2.47,-4.69, 1.58 and -8.68 respectively. Percentage of Matrix Effect of each Lot of IS was -2.99, -0.17, -0.21, -1.60, -3.48 and -3.44 respectively. The percentage CV of aqueous LQC for Analyte and IS were 0.90 and 0.71 respectively.

Calibration Curve: The accuracy of back calculated concentration of calibration standards ranges from 97.16% to 101.29%. The Percentage CV of back calculated concentration of calibration standards ranges from 1.05% to 4.97%.The coefficient of linear correlation (r2) is more than 0.99.

Precision and Accuracy: Accuracy for LOQQC ranges from 94.10% to 111.07% and for LOQ, MQC & HQC ranges from 90.98% to 104.45% in the intraday batches. Precision for LOQQC ranges from 3.07% to 5.31% and for LOQ, MQC & HQC ranges from 2.14% to 5.61% in the intraday batches. Accuracy for LOQQC was 102.20% and for LOQ, MQC & HQC ranges from 92.79% to 98.90% in the interday batches. Precision for LOQQC was 8.17% and for LOQ, MQC & HQC ranges from 2.61% to 6.02% in the Inter day batches.

Stability:

Freeze thaw stability: Mean Accuracy of comparison QC samples for LQC and HQC were 96.70% and 93.64% respectively. Mean Accuracy of QC samples at -20^{O}C for LQC and HQC were 100.68% and 95.96% respectively. Mean Accuracy of QC samples at -70^{O}C for LQC and HQC were 99.26% and 94.70% respectively. %CV of comparison QC samples for LQC and HQC were 3.82% and 3.11% respectively. %CV of QC samples at -20^{O}C for LQC and HQC were 4.36% and 2.67% respectively. %CV of QC samples at -70^{O}C for LQC and HQC were 3.09% and 1.73% respectively. Mean Accuracy of stability samples at -20^{O}C against comparison QC samples for LQC and HQC were 104.12% and 102.48% respectively. Mean Accuracy of stability samples at -70^{O}C against comparison QC samples for LQC and HQC were 102.65% and 101.13% respectively.

Short Term Stability: Mean Accuracy of comparison QC samples for LQC and HQC were 94.92 and 96.18 respectively. Mean Accuracy of stability QC samples for LQC and HQC were 101.37% and 96.06% respectively. %CV of comparison QC samples for LQC and HQC were 3.37% and 3.48% respectively. %CV of stability QC samples for LQC and HQC were 4.38% and 3.76% respectively. Mean Accuracy of stability samples at bench top for 6.20 hours against comparison QC samples for LQC and HQC were 106.80% and 99.87% respectively.

In Injector Stability: Mean Accuracy of comparison QC samples for LQC and HQC were 96.70% and 93.64%respectively.Mean Accuracy of stability QC samples for LQC and HQC were 98.95% and 95.24% respectively. %CV of comparison QC samples for LQC and HQC were 3.82% and 3.11% respectively. %CV of stability QC samples for LQC and HQC were 5.40% and 1.93% respectively. Mean Accuracy of stability samples compared with the bulk spiked comparison quality control sample about 35

hours for LQC and HQC were 102.33% and 101.71% respectively.

Long Term Stability: Mean Accuracy of comparison QC samples for LQC, MQC and HQC were 101.33%, 108.09% and 92.72% respectively. Mean Accuracy of QC samples at -70ºC for LQC, MQC and HQC were 103.23%, 94.49% and 95.86% respectively. Mean Accuracy of QC samples at -20^{o}C for LQC, MQC and HQC were 103.88%, 97.37% and 95.10% respectively. %CV of comparison QC samples for LQC, MQC and HQC were 8.76%, 1.18% and 5.59% respectively. %CV of QC samples at -70 ^{o}C for LQC, MQC and HQC were 4.11%, 4.28% and 6.68 % respectively. %CV of QC samples at -20^{o}C for LQC and HQC were 10.32%, 6.86% and 5.91% respectively. Mean Accuracy of stability samples at -70^{o}C against comparison QC samples for LQC, MQC and HQC were 101.88%, 87.42% and 103.38% respectively. Mean Accuracy of stability samples at -20^{o}C against comparison QC samples for LQC, MQC and HQC were 102.52%, 90.08% and 102.56% respectively.

Ruggedness: Mean Accuracy of LOQQC, LQC, MQC and HQC were 111.07%, 101.39%, 104.45% and 90.88% respectively. Precision for LOQQC, LQC, MQC and HQC were 5.31, 5.61, 4.85 and 2.42 respectively.

CONCLUSIONS: In summary, the method we have developed and validated is a sensitive, specific, and reproducible LC-MS-MS assay to quantify Montelukast using internal standard. This method meets the requirements and provides a high degree of accuracy, sensitivity and specificity using liquid chromatography and detection by electrospray tandem mass spectrometry. From the results of all the validation parameters, we can conclude that the present method can be useful for bioavailability/bioequivalence studies for determination of Montelukast with desired precision and accuracy.

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