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METHOD DEVELOPMENT FOR ESTIMATION OF MONTELUKAST SODIUM IN PLASMA BY LCMS/MS AND ITS VALIDATION

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Abstract:

The purpose of this investigation was to develop a rapid, simple, sensitive, and selective LC-MS/MS method for the quantitative estimation of Montelukast sodium in less volume of human plasma using less volume of blood. It is also expected that this method would provide an efficient solution for pharmacokinetic, bioavailability, and/or bioequivalence studies of Montelukast sodium. The precision and accuracy for Montelukast at this concentration was found to be 3.10% and 97.24%. The method has been found to be reproducible by performing three Precision and Accuracy (P&A) batches consisting of one intra day batch and two inter day batches.A sensitive method that is precise and accurate over a linear assay range of of 5.032ng/mL to 60.2362ng/mL of the drug has been validated for the determination of Montelukast in Human plasma using LC-MS/MS Method. Use of stable labeled isotopes as internal standards helped us to obtain the consistent and reproducible results. Also, the method showed no matrix effect and limited variability in recovery between analyte and IS. The method utilized only 100 µL of plasma for sample processing. A simple SPE technique with direct injection (avoids drying, evaporation and reconstitution steps) for sample preparation, thereby significantly reduces the sample processing time. The total run time per analysis of each sample is 3.0 min which allows analysis of more samples in a single day.

Keywords: *LC-MS/MS, Montelukast sodium, Precision , Accuracy*

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INTRODUCTION:

Montelukast sodium is an orally administered drug of choice in the treatment of asthma in adults and children. It is a potent, selective and orally acting leukotriene receptor antagonist used in the prophylaxis and treatment of asthma by inhibiting physiological actions of the cysteinyl leukotrienes (LTC4, LTD4and LTE4). Montelukast sodium has a biological half-life of about 2.5-5.5 hrs and 64 % bioavailability. Development of pulse release formulation of Montelukast sodium can be advantageous, that can provide specific lag time and increase compliancy of the dosage form towards patient's side[1].

In the present research work, we have attempted to develop a novel dosage form by using a chronopharmaceutical approach for the treatment of nocturnal asthma using Montelukast sodium as a model drug. The aim was to have a lag time of 5 h, i.e., the system is to be taken at bed time (9 pm) and is expected to release the drug after a period of 5 h, i.e., at 2 am. Literature evidence shows that the peak plasma concentration of Montelukast sodium is reached approximately 2 h after oral administration. Therefore, the drug concentration would be at its maximum level, when asthma attacks are more prevalent, i.e., at 4 am [2-4]. A pulsatile dosage form, taken at bedtime with a programmed start of drug release in the early morning hours, can prevent a sharp increase in the incidence of asthmatic attacks, during the early morning hours (nocturnal asthma), a time when the risk of asthmatic attacks is the greatest. Hence in the present study, pulsatile drug delivery system of Montelukast sodium was compared with that of Marketed Montelukast sodium SR formulation.

MATERIALS AND METHODS:

Estimation of Montelukast sodium in plasma (LC-MS/MS method[5]:

Chromatographic conditions

A summary of the chromatographic and mass spectrometric conditions is as follows:

HPLC : Shimadzu Nxera X2
Mass : Shimadzu LCMS 8050
Ion source : Turboion spray
Polarity : Positive ion mode.
Detection ions:
Montelukast sodium: 586.30*amu (parent), 422.20* amu (product)
Montelukast sodium –D6: 592.20* amu (parent), 427.20* amu (product)
Column : Agilent Zorbax XDB, C18, 5µm, 4.6x50mm,
Column oven temperature: 40.0 °C
Peltier temperature : 15 °C
Mobile phase : 10Mm ammonium formate: Acetonitrile (30:70)
Flow rate : 1.000ml/min.
Volume of injection : 10 µl
Retention times : Montelukast sodium: 0.2 to 1.70 minutes
Montelukast sodium –D6: 0.7 to 1.70 minutes
Run time : 3.50 minutes

Preparation of working standard solutions:

1. Preparation of Montelukast sodium stock solution:

Montelukast sodium working standard equivalent to5 mg of Montelukast sodium was weighed and transferred in to a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solution was calculated by considering the purity of Montelukast sodium. The solution was labeled and stored in a cold room at 2-8°C. The stock solution was diluted methanol to get a concentration about 1µg/ml.

2. Preparation of Montelukast sodium –D6 as internal standard stock solution:

Montelukast sodium –D6 working standard equivalent to 5 mg of Montelukast sodium –D6 was weighed and transferred into a 5 ml volumetric flask and dissolved in methanol. The solution was made up to the volume with methanol. The concentration of resulting solutions was calculated by considering the purity of Montelukast sodium –D6. The solutions were labeled and stored in a cold store at 2-8°C.

2. Calibration curve standards:

1. Preparation of stock dilutions of standard Montelukast sodium solution:

Stock dilutions of Montelukast sodium ranging from 20 ng/ml to 20000 ng/ml were prepared with 60% methanol using dilutions of main stock solution prepared for calibration curve standards.

2. Spiking of plasma for calibration curve standards:

Concentrations of Montelukast sodium ranging from 1 ng/ml to 1000 ng/ml were prepared with plasma and were labeled as CC1 to CC8. The calibration curve standards were prepared fresh for each validation run.

3. Sample preparation:

Step 1: Blank, calibration curve standards and the subject samples were withdrawn from the deep freezer and allowed them to thaw. The thawed samples were vortexed to ensure complete mixing of the contents. To 3 ml of plasma sample in a RIA vial, 30µl of Montelukast sodium –D6 (3µg/ml) was added. To plasma blank and pre-dose (0.0hr), 50 ul of

60%methanol in water solution was added. The samples were vortexed to ensure complete mixing of contents

Step 2: Approximately 2.5 ml of dichloromethane in diethyl ether solution was added and centrifuged for 10 minutes at approximately 4000 rpm at 20°C and the supernatant (organic layer) was transferred into another RIA vial. The organic layer was evaporated under a stream of nitrogen gas at 45°C. The residue was reconstituted with 0.3 ml of reconstitution solution and vortexed. The samples were transferred in auto-injector vials and were loaded in to auto sampler. 10 ul of sample was injected onto LC-MS/MS system. Analyte Concentrations of stock dilutions of standard Montelukast sodium –D6 solution with plasma were shown in Table 1.

4. Data processing

The chromatograms were obtained by using the computer-based lab solution software, version 75.8 supplied by the Schimadzu Corporation. The concentrations of the unknown samples have to be calculated from the equation using regression analysis of spiked plasma calibration standard with $1/x^2$ as weighting factor.

y = mx + c; Where, y = Ratio of Montelukast sodium peak area and Montelukast sodium –D6 peak area (Analyte area / ISTD area); x = concentration of Montelukast sodium; m = slope of the calibration curve; c = y-axis intercept value. Linear regression analysis equation of stock dilutions of standard Montelukast sodium solution with plasma is y = 0.0185x + 0.0023

Method validation:

Method validation was performed following ICH specifications for specificity, range of linearity,accuracy, precision and robustness.

System suitability [6]:

System performance parameters like retention time, number of theoretical plates, tailing factor, resolution were calculated by injecting standard solutions for six times. The resultant results were compared with the standard limits as per guidelines.

Specificity[6]:

It is the ability of a method to discriminate between the analyte of interest and other components that are present in the sample. These studies are to check the interferences in the optimized method. To assess the method specificity, blank and placebo were injected into HPLC system under optimized conditions. There should not be any interfering peak in the blank or placebo chromatograms at the retention times of the selected drugs.

Linearity[6]:

The linearity of the method was obtained by preparation of the calibration standards of six different concentrations in 6 replicates. The calibration curve plots for TEN & MET were obtained by plotting the peaks areas on y-axis and concentrations on x-axis over the concentration ranges of 5-30 µg/ml for TEN and 125-750 µg/ml for MET. The correlation coefficient should be greater than 0.99.

Accuracy[6]:

The accuracy of the method was assessed by recovery experiments by adding a known quantity of pure standard drug into the sample solution and recovering the same in terms of its peak areas. The sample was spiked with standard at levels of 50%, 100% and 150% of test concentrations. The resultant spiked sample was assayed in triplicate. The %recovery for each level should be in between 98%-102%.

Precision[6]:

It is the degree of closeness of agreement between the series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions. It is expressed in terms of standard deviation (SD) or relative standard deviation (RSD). Precision may be measure of either degree of repeatability or reproducibility of the analytical method.

Method precision [7]:

Sample solutions were injected under optimized conditions for six times on six different days and their peak areas were recorded. %RSD for the peak areas of 6 standard injection results should not be greater than 2.

Intermediate precision[7]:

Six replicates of sample solutions were injected under optimized conditions on the same day and their peak areas were recorded. %RSD for the peak areas of 6 replicate injection results should not be greater than 2.

Ruggedness:

The ruggedness of the method was determined by carrying out the experiment on different instruments, by different operators and using different columns of similar types.

Robustness [8]:

The robustness of the method was determined by making small deliberate changes in the method like flow rate, mobile phase ratio & temperature. But, one

should not find remarkable change in the results and the obtained results should be within range as per ICH guidelines.

Effect of variation of flow[9]:
Sample was analyzed at 0.9 ml/min & 1.1 ml/min flow rate instead of 1.0 ml/min, remaining conditions are kept constant.

Effect of variation of temperature[10]:
Temperature of 25°C and 35°C was maintained instead of 30°C. Samples were injected in triplicate & chromatograms were recorded.

LOD & LOQ[10]:
LOD is the smallest concentration that can be detected but not necessarily be quantified as an exact value. It is calculated using formula

LOD = 3.3 σ/S; where, σ = S.D; S =Slope

LOQ is the lowest amount of analyte in the sample that can be quantitatively determined with precision & accuracy

RESULTS AND DISCUSSION:

The LC-MS/MS methods were highly sensitive and suitable for the detection of drug in plasma even in low concentrations. Calibration curves were constructed from blank sample (plasma sample processed without IS), blank+IS samples and eight point calibration standards for Montelukast sodium in plasma.Six lots of Blank Human plasma were chosen to evaluate the slectivity of the method. Two replicates from each of those 6 lots of Montelukast sodium free blank human plasma samples spiked with Montelukast sodium at LLOQ concentration.Average interference response obtained at Montelukast sodium Retention Time with free blank human plasma is = 10% of the average response obtained from the corresponding lot of human plasma containing Montelukast sodium at LLOQ concentration along with IS.The lowest limit of reliable quantification for Montelukast in human plasma was set at the concentration of the LLOQ, 5.032ng/mL. The precision and accuracy for Montelukast at this concentration was found to be 3.10% and 97.24%. No statistical outlier was found. The results were given in Table: 6.8.

Matrix effect

The precision for IS normalized matrix factor at LQC and HQC level was found to be 0.69% and 0.61%, respectively. Each calibration curves was analyzed individually by using least square weighted (1/X2) linear regression. All the curves were forced through zero. Back calculation were made from the calibration curves to determine the concentration of Montelukast in each calibration standards. The calibration line was linear in the range of 5.032ng/mL to 60.2362ng/mL of the drug as shown in Fig:6.14. A straight-line fit made through the data points by least square regression analysis showed a constant proportionality with minimal data scattering. The correlation coefficient (r^2) was greater than 0.99 and ranged from 0.9960 to 0.9972 for Montelukast. The co-efficient of correlation were found to be better than 0.99 for the all calibration curves analyzed.The method has been found to be reproducible by performing three Precision and Accuracy (P&A) batches consisting of one intra day batch and two inter day batches. Each analytical run in P&A consists of two replicates of Standards at LLOQ and ULOQ, and one replicate at other levels along with 6 replicates of QC at all levels. Intra day run is evaluated from the Precision and Accuracy of 6 replicates of QC samples at LLOQ, LQC, MQC and HQC levels from the first three accepted analytical runs individually.The precision of the assay was measured by the percent coefficient of variation for QC samples of Montelukast. The accuracy of the assay was measured by computing the ratio of the calculated mean values of the QC samples to their respective nominal values, expressed as percentage nominal.

The precision of room temperature(20 ± 5□C)stock solution stability of Montelukast at 0 and 6 hours was found to be 0.56% and 0.73%, respectively and percentage of stability was found to be 101.96%. The precision of room temperature (20 ± 5 □C) stock solution stability of Montelukast D_6 at 0 hours and 7 hours was 0.48% to 1.04%respectively and percentage of stability was found to be 102.33%. The precision of room temperature (20 ± 5^0C) spiking solution stability of Montelukast at 0 hours and 7 hours 0.30% to 0.56%, respectively and percentage of stability was found to be 102.77% and for Montelukast D_6 at 0 hours and 7 hours was 0.69% to 0.48%respectively and percentage of stability was found to be 102.67%.

The precision ranged from 2.06% to 2.59% and percentage of stability was found to be 93.88%.Refrigerated stock solution stability of Montelukast D_6 was carried out by injecting six replicates of stock dilution at final working concentration level. The stock solutions were found to be stable for 4 days. The precision ranged from 2.43% to 3.17% and percentage of stability was found to be 93.28%. Stability standard stock solution of Montelukast and Montelukast D_6 was

compared with fresh standard stock solution. Results demonstrate that the processed samples were stable for 51 hours. The percent nominal at 51 hours ranged from 91.96% to 109.15% and precision ranged from 0.95% to 1.86%.Montelukast was found to be stable up to 10 hours. The percent nominal ranged from 90.34% to 107.21% for 10 hours. The precision ranged from 0.68% to 2.06% for10 hours.The freeze-thaw quality control samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for the calculation of precision and accuracy. Montelukast result demonstrated freeze thaw stability. The percent nominal ranged from 90.64% to 107.39% for four freeze-thaw cycles and the precision ranged from 1.15% to 1.31%. No statistical outlier was found.Percentage of Stability was calculated against freshly spiked quality control samples. Montelukast percent nominal ranged from 98.59%to 102.14%and the precision ranged from 1.66% to 1.76%. No statistical outlier was found.Montelukast results demonstrate that the processed samples were stable for 50 hours at room temperature. The percent nominal at 50 hours ranged from 90.93% to 107.42% and precision ranged from 0.88% to 1.72%. No statistical outlier was found.The mean concentrations of reinjected QCs were compared against the mean of the QCs when injected for first time. The results demonstrate that the reinjected samples were stable for 44 hours. Montelukast percent nominal at 24 hours ranged from 92.86% to 97.13% and precision ranged from 0.76% to 2.94% and no statistical outlier was found for 0 and 44 hours.The mean overall recovery of Montelukast was 58.56% with a precision ranging from 1.00% to 5.17%. The mean recovery of internal standard Montelukast D_6 was 57.75% with a precision ranging from 4.25% to 5.08%.No statistical outlier was found.

Montelukast results demonstrate acceptable dilution integrity for two times and four times dilution. Montelukast precision and accuracy, for a dilution factor of 2 was 0.72% and 95.03%, respectively. Similarly, Montelukast precision and accuracy, for a dilution factor of 4 was 0.98% and 97.97%, respectively. No statistical outlier was found..

Table 1: Analyte Concentrations of Stock Dilutions of Standard Montelukast sodium Solution with Plasma

S.NO	Sample Name	Analyte Concentration (ng/ml)	Analyte peak area	IS Peak Area	Area Ratio	Calculated Concentration (ng/ml)	Accuracy (%)
1	RSS	-	680063	398176	1.71	92.4433	-
2	Plasma Blank	-	-	-	-	N/A	N/A
3	Blank+ISTD	0	-	437854	-	N/A	N/A
4	CC1	1	10233	491994	0.02	1.018	101.83
5	CC2	2	17152	451751	0.04	1.949	97.43
6	CC3	5	39177	430754	0.09	4.820	96.39
7	CC4	20	173105	447531	0.39	20.851	104.26
8	CC5	100	679807	388089	1.75	94.813	94.81
9	CC6	300	2116502	379538	5.58	302.076	100.69
10	CC7	700	5670606	415905	13.63	738.721	105.53
11	CC8	1000	6479070	354437	18.28	990.456	99.05

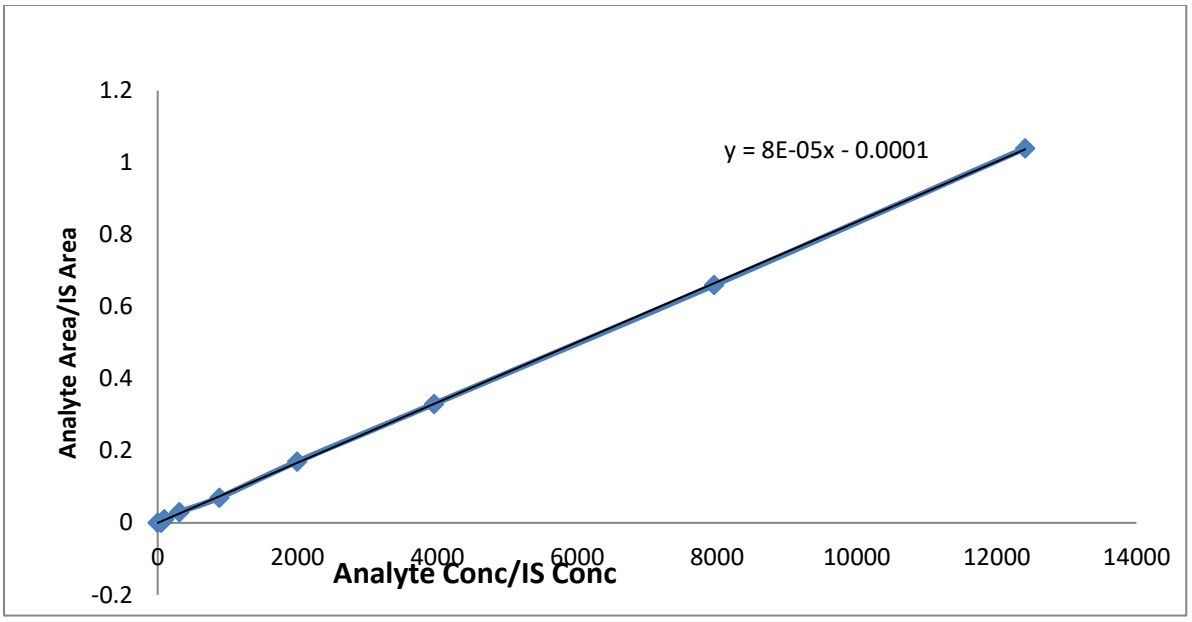

Figure 1: Calibration Curve for Estimation of Montelukast sodium in Plasma

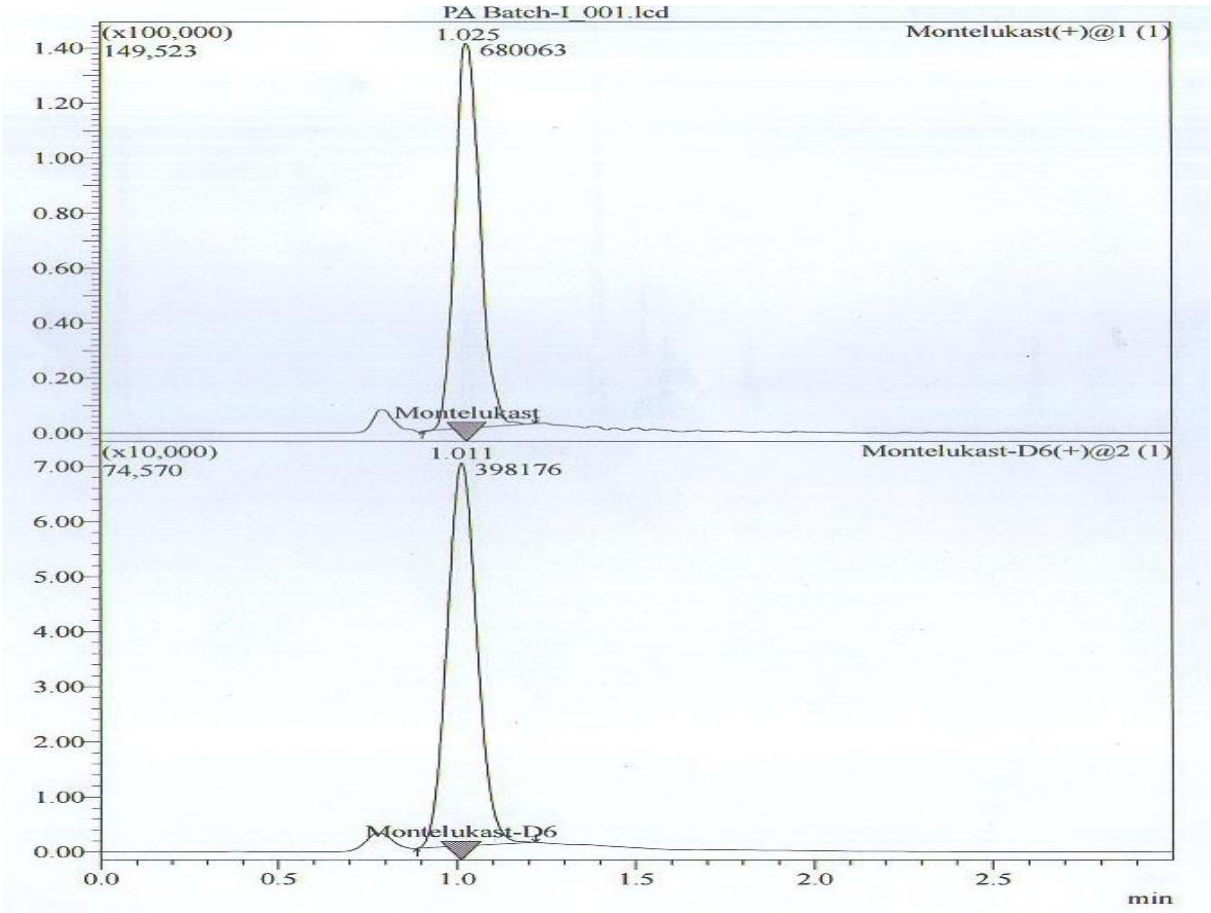

Figure 2 and 3: Chromatograms of reserve stock solution of Standard Montelukast sodium Solution with Plasma

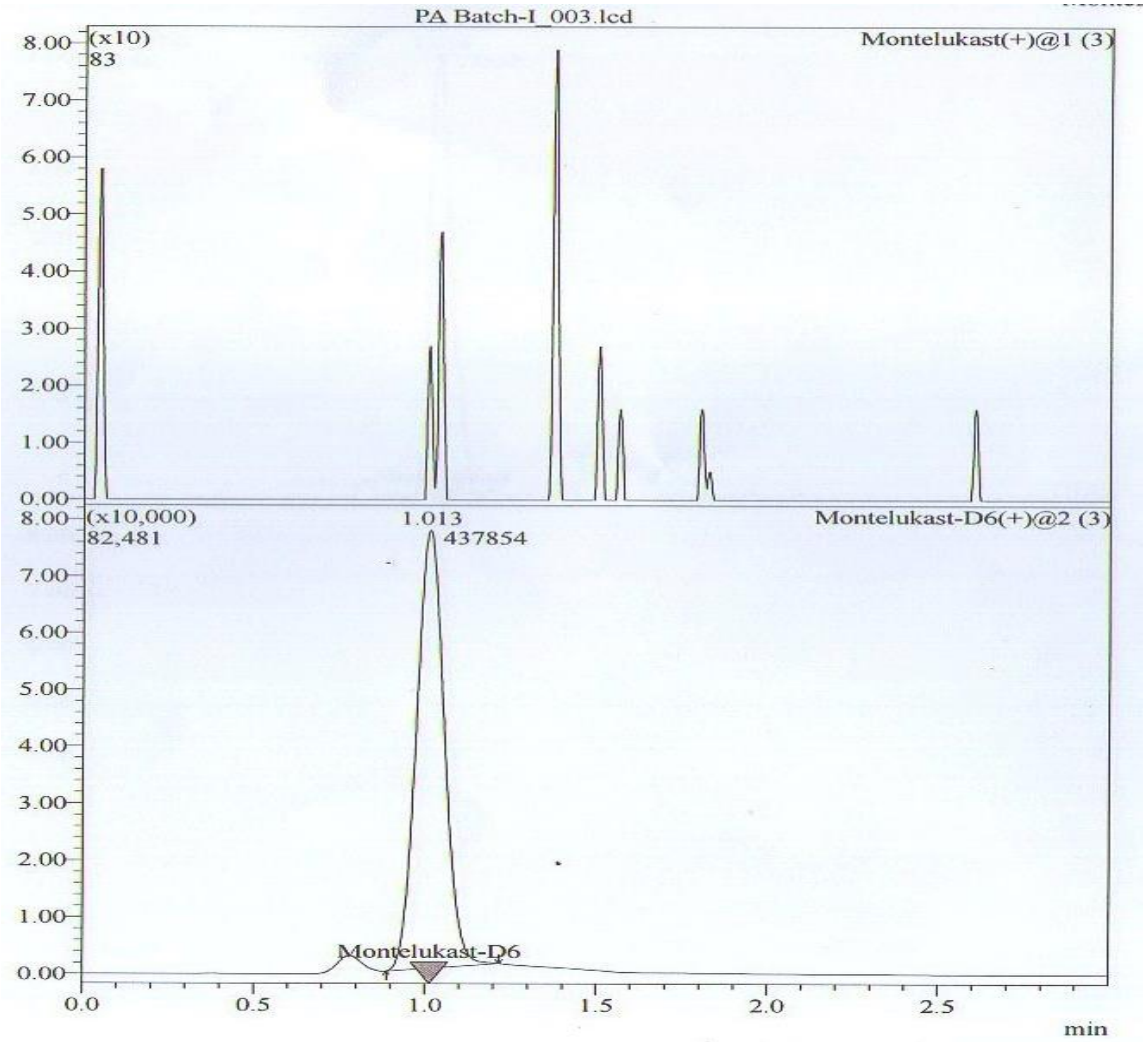

Figure 4: Chromatograms of Plasma blank and internal standard (Montelukast sodium D6**)**

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