AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

LC-MS/MS method development and validation of Montelukast in human plasma and its clinical application

Mahitej Yadav Katteboina[1], Nageswara Rao Pilli[1], Shobha Rani Satla*[1]
1. Center for Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad–500 085, India

ABSTRACT

In the present paper, the authors described a novel Liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of montelukast in human plasma using montelukast d6 as internal standard (IS). After solid phase extraction (SPE), the analyte and the IS were chromatographed on a C18 columns using a isocratic mobile phase composed of acetonitrile–5mM ammonium acetate (80:20, v/v) pumped at a flow rate of 0.8mL/min. The proposed method was validated in the range of 5.01–599.91 ng/mL as per the US FDA guidelines. Precision and accuracy results were calculated using five successful calibration curves. Analyte stability in true samples and in plasma samples under different conditions were established and results met the acceptance criteria. The chromatographic run time was set at 3 min, which makes the proposed method is high through put. The method was successfully applied to a pharmacokinetic study in healthy South Indian male subjects under fasting condition.

Keywords: Montelukast, Solid–phase extraction (SPE), LC–MS/MS, Method validation, Pharmacokinetics.

*Corresponding Author Email: mahitejyadavk@gmail.com
Received 01 June 2015, Accepted 07 June 2015

Please cite this article as: Katteboina MY *et al*., LC-MS/MS method development and validation of montelukast in human plasma and its clinical application. American Journal of PharmTech Research 2015.

INTRODUCTION

Montelukast is a specific cysteinyl leukotriene receptor antagonist, used for the treatment of bronchial asthma and seasonal allergic rhinitis. Leukotrienes are produced and released from proinflammatory cells and are highly potent broncho constrictors that lead to the development of asthma and associated symptoms [1-3].

A review of literature reveals that many LC-MS/MS methods [4-9] have been reported for determination of montelukast. Most of the LC-MS methods [4-8] reported so far were utilizes protein precipitation (PP) technique for the biological sample processing. PP is most likely to cause ion suppression, since the method fails to sufficiently remove endogenous compounds such as lipids, phospholipids, fatty acids, etc[10, 11]. Additionally, some methods[4-6] are having a long chromatographic run time (>4 min) which may not be suitable for high–throughput bioanalysis of montelukast. The method reported by Ezzeldi et al., 2014 [4] and Muppavarapu et al., 2014[7] are not specific for montelukast and it may create conflicts in final results due to improper characterization of selectivity. Another method reported by Bharathi et al., 2009[9] utilizes a time–cost sample preparation involving liquid–liquid (L–L) extract, evaporation, drying and reconstitution steps. The analytical method should satisfy the scientists in terms of simplicity, sensitivity, runtime, sample volume, time consumption and efficient extraction procedure.

The aim of the present study was to develop and validate a simple, rapid and sensitive LC-MS/MS method for the determination of montelukast in human plasma using isotope labeled compound montelukast d6 as internal standard. The analyte and the IS were extracted from 100 µL of human plasma using solid–phase extraction with no drying, evaporation and reconstitution steps. The proposed method was successfully applied to a pharmacokinetic study in humans.

MATERIALS AND METHOD

Standards and reagents

Reference standards of montelukast sodium (99.83% pure) and montelukast d6 sodium (99.62% pure) were obtained from Vivan Life sciences Limited (Mumbai, India). Formic acid and ammonium acetate (analytical grade) were purchased from Merck Ltd (Mumbai, India). HPLC grade acetonitrile and methanol were purchased from J.T. Baker (Phillipsburg, USA). LC-MS grade water was prepared by using Milli Q water purification system procured from Millipore (Bangalore, India). Blank human plasma samples were obtained from Deccan's Pathological Lab's (Hyderabad, India).

LC–MS/MS instrument and conditions

An HPLC system (Shimadzu, Kyoto, Japan) consisting of a binary LC–20AD prominence pump,

an auto sampler (SIL–HTc) and a solvent degasser (DGU–20A_3) was used for the study. Aliquot of 20 µL of the extracted samples were injected into the Zorbax Eclipse XDB-Phenyl column (75 mm x 4.6 mm, 3.5 µm), which was kept at 40 °C. An isocratic mobile phase consisting of a mixture of acetonitrile–5mM ammonium acetate (80:20, v/v) was used for the analysis. The mobile phase flow rate was set 0.80 mL/min. An API–4000 mass triple quadrupole spectrometer (MDS Sciex, Foster City, CA, USA) equipped with a Turboionspray ™ interface was used for the current study. Electro spray ionization (ESI) source temperature was maintained at 500 °C. The ion spray voltage was set at 5000 V. The source parameters viz. the nebulizer gas (GS1), auxiliary gas (GS2), curtain gas and collision gas were set at 45, 45, 30, and 8 psi, respectively. The compound parameters viz. the declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit potential (CXP) were 78, 35, 10, 23 V for montelukast and for the IS. Detection of the ions was carried out in the multiple–reaction monitoring mode (MRM), by monitoring the transition pairs of *m/z* 586.3 precursor ion to the *m/z* 422.3 for montelukast and *m/z* 592.3 precursor ion to the *m/z* 427.3 product ion for the IS. Quadrupoles Q1 and Q3 were set on unit resolution. The chromatographic data was processed by Analyst Software™ (version 1.6.1).

Preparation of stock and working solutions

A 1 mg/mL stock solutions of montelukast and Montelukast d6 were prepared in methanol. Two separate stock solutions were prepared for montelukast and used for the preparation of calibration curve (CC samples) standards and quality control (QC) samples, respectively. All the working solutions were prepared in a mixture of acetonitrile and water (60:40, v/v; diluent).

Calibrates in plasma were prepared at a concentration levels of 5.01, 10.02, 30.01, 60.02, 120.04, 240.09, 359.95, 479.93 and 599.91 ng/mL. Equally, quality control (QC) samples were also prepared at concentrations of 5.03 (lower limit of quantitation, LLOQ), 15.23 (low quality control, LQC), 91.19 (medium quality control, MQC1), 303.98 (MQC2) and 467.66 ng/mL (high quality control, HQC) as a single batch at each. All the bulk spiked samples were stored in deep freezer at –70±10 °C.

Sample processing protocol

An aliquot of 100 µL of thawed human plasma sample was mixed with 10 µL of the internal standard working solution (1002 ng/mL of montelukast d6). To this, 200 µL of 1% formic acid solution was added after vortex mixing for 10 s. The sample mixture was loaded onto an Orpheus C_{18} cartridge (100 mg/1 mL) that was pre–conditioned with 1.0 mL of methanol followed by 1.0 mL of water and 1.0 mL of 1% formic acid solution. The extraction cartridge was washed with 1.0 mL of 100mM ammonium acetate followed by 2.0 mL of water (1 mL each time). Analyte and the

IS were eluted with 0.5 mL of mobile phase. Aliquot of 20 µL of the extract was injected into the LC-MS/MS system.

Method validation parameters

A thorough and complete method validation of montelukast in human plasma was carried out as per US FDA guidelines [12]. The parameters determined were carryover test, selectivity, matrix effect, sensitivity, linearity, precision and accuracy, recovery, dilution integrity, and stability.

Pharmacokinetic study design

A pharmacokinetic study was conducted using montelukast 5 mg chewable tablet in 7 healthy male subjects under fasting condition. Ethics committee approved the protocol and the informed consent was obtained from each subject before commencement of the study. The subjects were fasted for 12 h prior to drug administration. Blood samples were at pre–dose and 0.5, 1, 1.25, 1.5, 1.75, 2, 2.25, 2.5, 2.75, 3, 3.5, 4, 5, 6, 8, 10, 12, 16, and 24 h, in K_2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA). The tubes were centrifuged at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –70 ± 10 °C till their use. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. The QC samples at low, middle 1, middle 2 and high concentration levels were also assayed in triplicate along with the study samples. WinNonlin software (Version 5.1) was used to analyze the plasma concentration–time profile of montelukast. As per the FDA recommendations, conducting the incurred sample reanalysis (ISR) is compulsory to validate the obtained *in-vivo* concentration data. Two samples from each subject near to Cmax and the elimination phase (A total of 14 samples) were selected from six subjects for ISR. The ISR values were compared with the initial values. The percent change deviation allowed is ±20% [13, 14].

RESULTS AND DISCUSSION

Mass spectrometry

Mass spectrometric parameters were optimized by infusing the 100 ng/mL solution of analyte and the IS in to the ESI source. High intensities were obtained in positive mode than the negative mode. Source and compound dependent parameters were suitable altered to obtain the high and reproducible response. Protonated form of analyte and IS, $[M+H]^+$ ion was the parent ion in the Q_1 spectrum and was used as the precursor ion to obtain Q_3 product ion spectra. The most sensitive mass transition was observed from m/z 586.3 to 422.3 for montelukast and from m/z 592.3 to 427.3 for the IS. The dwell time for each transition was 200 ms. As earlier publications have discussed the details of fragmentation patterns of montelukast [6] and IS [6], we are not presenting the

data pertaining to this. The MRM technique was chosen for the assay development as it provides inherent selectivity and sensitivity.

Method development

Mobile phase composition plays an important role to obtain good chromatography and better resolution of analyte from the endogenous components. Hence different options were evaluated using organic solvents like methanol and acetonitrile in combination with acidic buffers namely ammonium acetate and ammonium formate in varying strength. It was found that a combination of acetonitrile–5mM ammonium acetate (80:20, v/v) as the mobile phase was most appropriate to give best sensitivity, efficiency and peak shape. The mobile phase was operated at a flow rate of 1.0 mL/min. Among the different columns tested, Zorbax Eclipse XDB-Phenyl (75 mm × 4.6 mm, 3.5 µm) column gave good peak shape and response even at LLOQ level for the analyte. The retention time of analyte and the IS were low enough (1.30 and 1.30 min) allowing a short run time of 3.0 min.

The reported procedures have employed PP [4-8] and LLE [9] with evaporation and reconstitutions steps to extract montelukast from biological samples. But the as a purpose to develop an effective and sensitive method with minimum or no matrix effect, SPE was tested. SPE was checked with Oasis HLB, Strata X polymeric sorbent and Orpheus C_{18} cartridges in presence of ammonium acetate and formic acid in different strengths. Reliable and reproducible recovery (~80%) was obtained with Orpheus C_{18} cartridges at all QC levels for the analyte. Thus, the simple SPE procedure was employed for the sample preparation in the present work and provided high recovery for the analyte. Addition of 1% formic acid to the plasma samples helped in achieving consistent and reproducible recovery.

A good internal standard should mimic the analyte in as many ways as possible. Use of stable isotope–labeled drugs as internal standards proves to be helpful when a significant matrix effect is possible and to increase assay precision and limit variable recovery between analyte and the IS. Hence, in the present work montelukast stable labeled isotope montelukast d6 was used as internal standard and found to be best for the present purpose.

Carryover effect

Carryover test was performed to check the possible carryover in subsequent runs after injection of highest concentration of analyte (ULOQ; upper limit of quantitation). Results reveals that there was no significant carryover effect in the blank samples after injection of ULOQ sample.

Selectivity and chromatography

A total of eight blank plasma lots from the different sources (6 were normal and one lipemic and

one haemolyzed) were screened for the selectivity experiment. As shown in Figure 1A no significant direct interference in the blank plasma traces was observed from endogenous substances in drug–free human plasma at the retention time of the analyte and the IS. Figure 1B depicts a representative ion–chromatogram for the LLOQ (CS–1) sample (5.01 ng/mL). A representative chromatograms resulting from the analysis of subject blank plasma sample along with the IS and 3.00 h subject plasma sample after the administration of a 5 mg chewable tablet of montelukast is shown in the Figure 2.

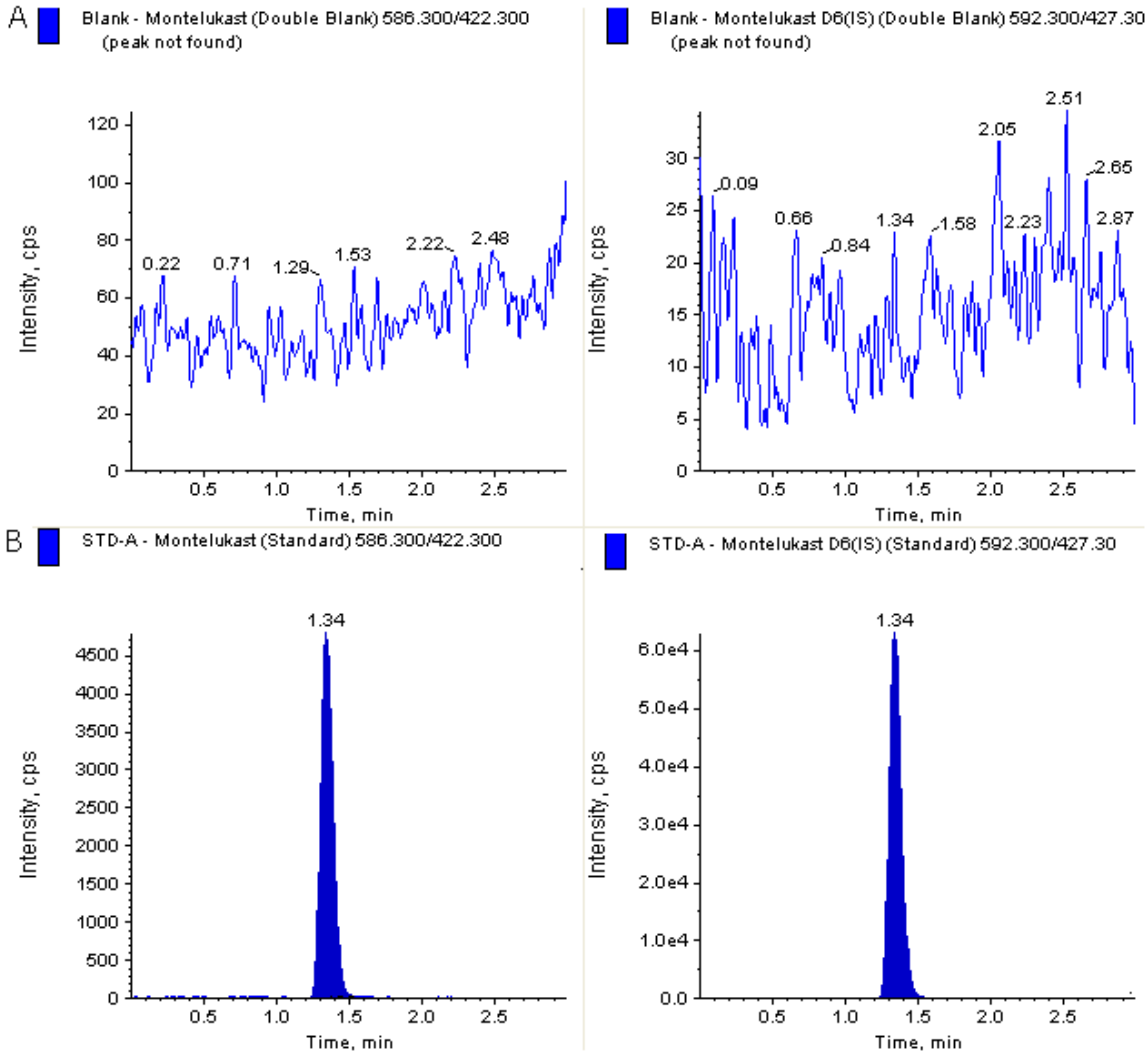

Figure 1. Typical MRM chromatograms of montelukast (left panel) and IS (right panel) in human blank plasma (A), and human plasma spiked with IS (B), a LLOQ sample along with IS (C).

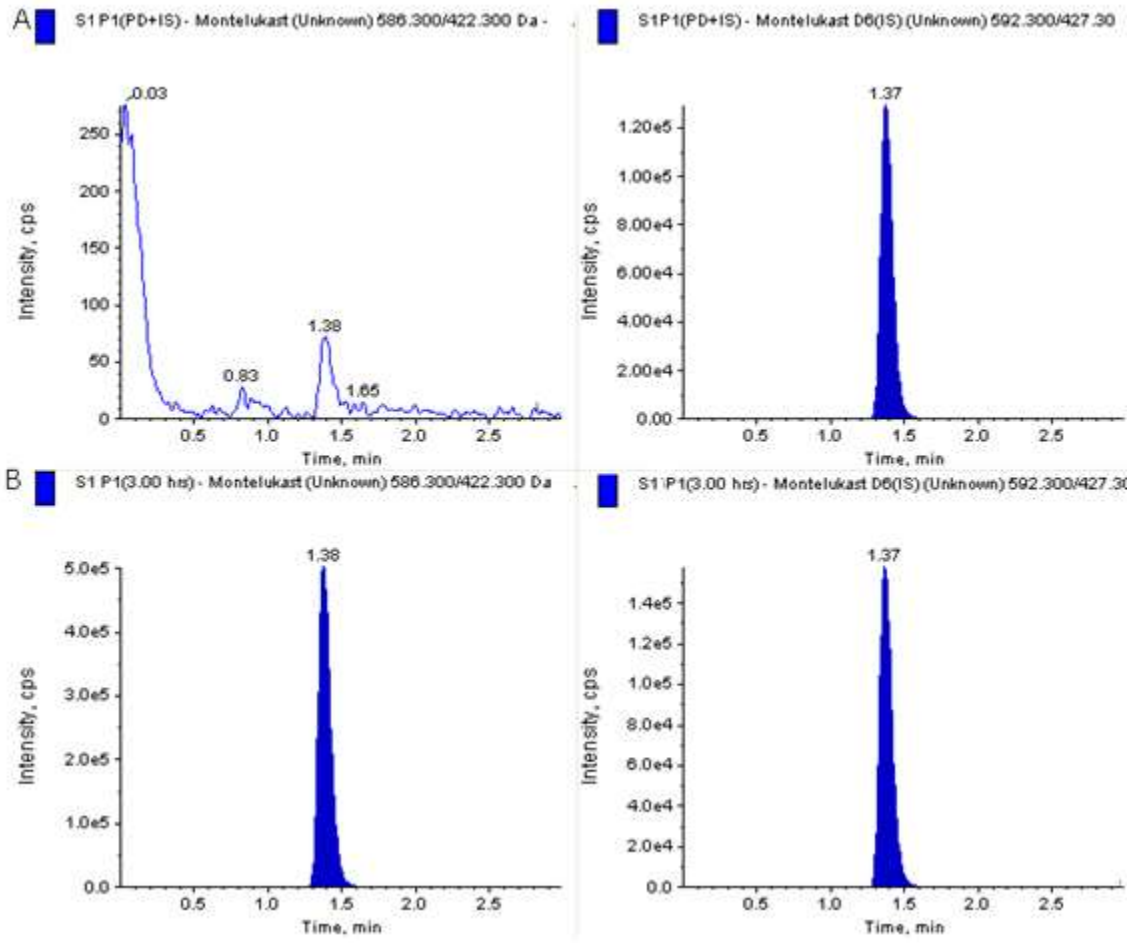

Figure 2. MRM chromatograms resulting from the analysis of subject blank plasma sample along with the IS (A) and 3.00 h subject plasma sample (B), after the administration of a 5 mg single dose of montelukast chewable tablet. The sample concentration was determined to be 254.68 ng/mL.

Matrix effect and sensitivity

Matrix effect, expressed as IS normalized matrix factor (MF) was assessed by comparing the mean area response of post–extraction spiked samples with mean area of aqueous samples (neat samples) prepared in mobile phase solutions at LQC and HQC levels.

IS–normalized MF was calculated using the below formula:

$$\text{IS normalized matrix Factor} = \frac{\text{Peak response area ratio in presence of matrix ions}}{\text{Mean peak response area ratio in absence of matrix ions}}$$

The %CV for IS normalized matrix factor at LQC and HQC level was found to be 1.15% and 0.93%, respectively and IS normalized factor was 1.005 for LQC and 0.992 for HQC. The results indicates no significant matrix effect was found in all the six lots of human plasma for the analyte.

The lowest limit of reliable quantification (LOQ) for the present method was set at 5.01 ng/mL. At this concentration, the precision and accuracy of analyte was found to be 1.81 and 103.94%, respectively.

Linearity, precision and accuracy

A total of five successful calibration curves were generated over the concentration range of 5.01–599.91 ng/mL for montelukast. After comparing the two weighting models (1/x and $1/x^2$), a regression equation with a weighting factor of $1/x^2$ of the drug to the IS concentration was found to produce the best fit for the concentration–detector response relationship. The mean correlation coefficient of the weighted calibration curves generated during the validation was ≥ 0.99.

The results for intra–day and inter–day precision and accuracy in plasma quality control samples are summarized in Table 1. The intra–day and inter day precision deviation values were all within 15% of the relative standard deviation (RSD) at low, middle and high quality control level, whereas within 20% at LLOQ QCs level. The intra–day and inter–day accuracy deviation values were all within 100 ± 15% of the actual values at low, middle, and high quality control level, whereas within 100± 20% at LLOQ QCs level. The results revealed good precision and accuracy.

Table 1:Precision and accuracy data for montelukast

Quality control	Run	Concentration found Mean±SD (ng/mL)	Precision (%)	Accuracy (%)
Intra–day variations (*n*=12 at each concentration)				
LLOQ		4.57 ± 0.10	2.13	90.84
LQC		14.58 ± 0.90	6.19	95.72
MQC1		84.29 ± 4.20	4.99	92.43
MQC2		280.17 ± 12.29	4.39	92.17
HQC		426.68 ± 10.74	2.52	91.24
Inter–day variations (*n*=30 at each concentration)				
LLOQ		5.08 ± 0.94	18.56	101.06
LQC		14.62 ± 0.54	3.71	95.99
MQC1		85.15 ± 3.57	4.19	93.37
MQC2		279.04 ± 9.79	3.51	91.80
HQC		427.48 ± 15.33	3.59	91.41

Nominal concentrations of LLOQ, LQC, MQC1, MQC2 and HQC are 5.03, 15.23, 91.19, 303.98 and 467.66 ng/mL, respectively.

Recovery and dilution integrity

Montelukast recovery was determined at LQC, MQC2 and HQC levels, whereas for the IS was determined at 1002 ng/mL. The mean overall recovery (with precision range) of montelukast was 83.77±1.78% (1.63–8.22%) and the recovery of IS was 80.00% with the precision range of 0.97–11.35%.

Dilution integrity experiment was performed to extent the ULOQ suitable for higher doses of montelukast. The upper concentration limit of montelukast can be extended to 950.90 ng/mL for by 1/2 and 1/4 dilutions with screened human blank plasma. The precision (%CV) for dilution integrity of 1/2 and 1/4th dilution was found to be 0.62% to 0.44%, while the accuracy results were found to be 96.18% and 99.29%, respectively.

Stability studies

Analyte stability at various conditions was evaluated. In the different stability experiments carried out viz. bench top stability (11 h), autosampler stability (52 h), wet extract stability at 2–8 °C (46 h), repeated freeze–thaw cycles (4 cycles), reinjection stability (39 h) and long term stability at –70 °C for 66 days the mean % nominal values of the analyte were found to be within ±15% of the predicted concentrations for the analyte at their LQC and HQC levels (Table 2). Therefore, the results were found to be within the acceptable limits during the entire validation.

Table 2 Stability data for montelukast in plasma (*n*=6)

Stability test	QC (spiked concentration (ng/mL)	Mean ± SD (ng/mL)	Precision (%)	Accuracy/ Stability (%)
Process[a]	15.23	14.53 ± 0.33	2.27	95.40
	467.66	427.20 ± 2.22	0.52	91.35
Process[b]	15.23	14.78 ± 0.18	1.19	97.04
	467.66	422.35 ± 4.76	1.13	90.31
Bench top[c]	15.23	14.48 ± 0.18	1.21	95.08
	467.66	426.10 ± 3.84	0.90	91.11
FT[d]	15.23	14.77 ± 0.13	0.86	96.99
	467.66	424.88 ± 4.30	1.01	90.85
Reinjection[e]	15.23	14.88 ± 0.18	1.19	97.73
	467.66	425.78 ± 5.63	1.32	91.04
Long–term[f]	15.23	15.81 ± 0.17	1.07	103.81
	467.66	475.56 ± 1.74	0.37	101.69

[a] after 52 h in autosampler at 10°C; [b] after 46 h at 2–8°C; [c] after 11 h at room temperature; [d] after 4 freeze and thaw cycles; [e] after 39 h of Reinjection; [f] at –70°C for 66 days

Pharmacokinetic study and incurred samples reanalysis

The validated method was then applied to determine the *in-vivo* plasma contraptions obtained from a pharmacokinetic study in humans (*n*=7). The obtained pharmacokinetic parameters were listed in the Table 3 and the results were comparable with the previous reports [6]. Figure 3 depicts the mean plasma concentration *vs* time profile of montelukast after administration of a single montelukast 5 mg chewable tablets under fasting condition. The obtained study data was authenticated by means of incurred sample reanalysis (ISR). The differences in concentrations obtained were less than 20% (Table 4), indicating good reproducibility of the present method.

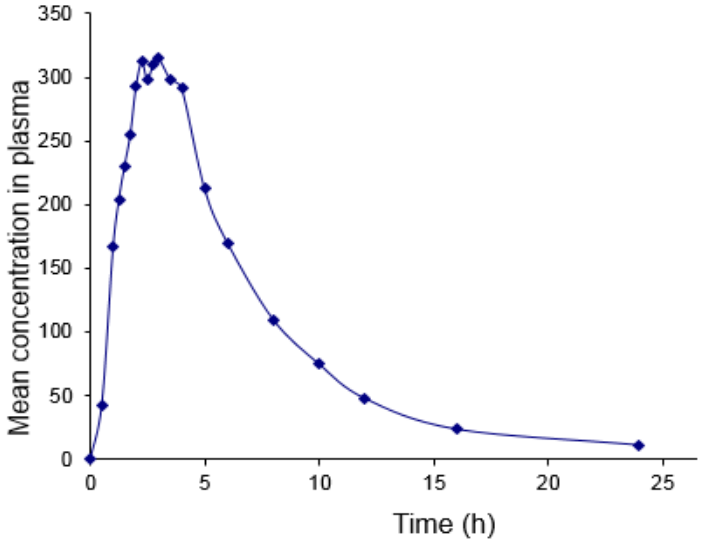

Figure 3. Mean plasma concentration–time profile of montelukast in human plasma following administration of montelukast (5 mg chewable tablet) to healthy volunteers (*n*=7).

Table 3: Pharmacokinetic parameters of montelukast after single oral administration of 5 mg tablet to healthy South Indian male subjects (*n*=6, Mean ± SD).

Parameter	Estimated value
C_{max} (ng/mL)	362.50 ± 43.49
t_{max} (h)	2.79 ± 0.85
AUC_{0-t} (ng h/mL)	2195.22 ± 390.27
AUC_{0-inf} (ng h/mL)	2285.65 ± 427.98
$t_{1/2}$ (h)	4.09 ± 0.84

Table 4 : Incurred samples re–analysis data of montelukast.

Sample	Initial conc. (ng/mL)	Re–assay conc. (ng/mL)	Difference [a] (%)
1	300.12	321.21	-6.79
2	31.43	32.65	-3.81
3	315.25	299.23	5.21
4	23.62	25.94	-9.36
5	348.21	357.35	-2.59
6	19.97	20.31	-1.69
7	386.41	371.77	3.86
8	22.97	21.31	7.50
9	371.83	368.94	0.78
10	33.44	35.60	-6.26
11	322.00	349.93	-8.31
12	50.34	56.09	-10.81
13	385.94	386.01	-0.02
14	32.91	31.38	4.76

[a] Expressed as [(initial conc.−re–assay conc.)/average]×100%.

CONCLUSIONS

In conclusion, this paper describes a simple, rapid and specific LC–MS/MS method for the determination of montelukast in human plasma. 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. The method showed suitability for pharmacokinetic studies in humans. From the results of all the validation parameters, we can conclude that the developed method can be useful for bioavailability and bioequivalence (BA/BE) studies and routine therapeutic drug monitoring with the desired precision and accuracy.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge PCR Laboratories (Hyderabad, India) for providing necessary facilities to carry out this work.

REFERENCES

1. Jones TR, Labelle M, Belley M, Champion E, Charette L, Evans J, Ford-Hutchinson AW, Gauthier JY, Lord A, Masson P. Pharmacology of montelukast sodium (Singulair), a potent and selective leukotriene D4 receptor antagonist. Can J Physiol Pharmacol. 1995; 73(2):191-201.
2. Reiss TF, Chervinsky P, Dockhorn RJ, Shingo S, Seidenberg B, Edwards TB. Montelukast, a once-daily leukotriene receptor antagonist, in the treatment of chronic asthma: a multicenter, randomized, double-blind trial. Montelukast Clinical Research Study Group. Arch Intern Med. 1998; 158(11):1213-1220.
3. Nayak A. A review of montelukast in the treatment of asthma and allergic rhinitis. Expert Opin Pharmacother. 2004; 5(3) 679-686.
4. Ezzeldin E, Abo-Talib NF, Tammam MH, Shahat AA. Development and validation of LC/MS/MS method for the simultaneous determination of montelukast, gliclazide, and nifedipine and its application to a pharmacokinetic study. Chem Cent J. 2014; 8(1):17.
5. Ezzeldin E, Tammam MH, AboTalib NF. Development of an LC-tandem mass spectrometry method for the separation of montelukast and its application to a pharmacokinetic study in humans. Drug Res (Stuttg). 2014; 64(11): 617-22.
6. Challa BR, Awen BZ, Chandu BR, Khagga M, Kotthapalli CB. Method development and

validation of montelukast in human plasma by HPLC coupled with ESI-MS/MS: application to a bioequivalence study. Sci Pharm. 2010; 78(3): 411-422.

7. Muppavarapu R, Guttikar S, Rajappan M, Kamarajan K, Mullangi R. Sensitive LC-MS/MS-ESI method for simultaneous determination of montelukast and fexofenadine in human plasma: application to a bioequivalence study. Biomed Chromatogr. 2014; 28(8):1048-1056.
8. Papp R, Luk P, Mullett WM, Kwong E. A rapid and sensitive method for the quantitation of montelukast in sheep plasma using liquid chromatography/tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2007; 858(1-2): 282-286.
9. Bharathi DV, Hotha KK, Jagadeesh B, Mullangi R, Naidu A. Quantification of montelukast, selective cysteinyl leukotriene receptor (CysLT1) antagonist In human plasma by liquid chromatography-mass spectrometry- validation and its application to a human pharmacokinetic study. Biomed Chromatogr. 2009; 23(8): 804–810.
10. Kole PL, Venkatesh G, Kotecha J, Sheshala R. Recent advances in sample preparation techniques for effective bioanalytical methods. Biomed Chromatogr. 2011;25(1/2):199–217.
11. Van Eeckhaut A, Lanckmans K, Sarre S, Smolders I, Michotte Y. Validation of bioanalytical LC–MS/MS assays: evaluation of matrix effects. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877(23): 2198–2207.
12. US DHHS, FDA and CDER. Guidance for Industry: Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine, 2001. Available at: http://www/ fda.gov/cder/guidance/index.htm.
13. de Boer T, Wieling J. Incurred sample accuracy assessment: design of experiments based on standard addition. Bioanalysis. 2011; 3(9):983-992.
14. Viswanathan CT, Bansal S, Booth B, DeStefano AJ, Rose MJ, Sailstad J, Shah VP, Skelly JP, Swann PG and Weiner R. Quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays. Pharm Res 2007; 24(10): 1962–1973.