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RESEARCH ARTICLE
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Bioanalytical Method Development and Validation of Stabiliity Indicating Lc-Ms/Ms Method to Determine Montelucast in Human Plasma

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ABSTRACT

Objective: A rapid and sensitive liquid chromatography-tandem mass spectrometric (LC-MS/MS) assay method was developed and fully validated for the determination of Montelucast in human plasma.

Materials and Methods: Montelucast D6 Sodium salt was used as an internal standard (IS). Analytes and the internal standard were extracted from human plasma by solid-phase extraction technique using Oasis HLB, Oasis Max, Varian Bond Elute Plexa, Orochem cartridges. The reconstituted samples were chromatographed on a ZORBAX Eclipse XDB Phenyl (4.6 X 75 mm, 3.5µ) by using **Acetonitrile:** 5mM Ammonium acetate buffer (85:15 v/v) as the mobile phase at a flow rate of 1.0 mL/min.

Results and Discussion: Detection was carried out LC-MS/MS (API 3000) in negative ion mode. The calibration curves obtained were linear (R2-0.999) over the concentration range of 5.032 - 602.362 ng/mL for Montelucast.. The results of the intra- and inter-day precision studies were well within the acceptable limits. 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 D6 was 57.75% with a precision ranging from 4.25% to 5.08%.No statistical outlier was found.

Conclusion: The analyte were found to be stable of stability study. Developed and validated analytical method was found to be simple, rapid, specific, sensitive, precise and cost effective than reported methods. The method has been successfully applied to the investigation of a preclinical pharmacokinetic study with desired precision and accuracy along with high throughput.

Keywords: *Montelucast, liquid chromatography, MS/MS, Montelucast D6 sodium salt*

INTRODUCTION

Montelukast (Figure 1) is chemically known as 2-[1-[1(*R*)-[3-[2(*E*)-(7-chloroquinolin-2-yl)vinyl] phenyl]-3[2-(1-hydroxy-1-methylethyl)phenyl]propylsulfanylmethyl] cyclo propyl] acetic acid sodium salt It is a leukotriene receptor antagonist (LTRA) used for the maintenance treatment of asthma and to relieve symptoms of seasonal allergies[1,2]. Itis usually administered orally. Montelukast is a $CysLT_1$ antagonist[3,4], that it blocks the action of leukotriene D4 (and secondary ligands LTC4 and LTE4) on the cysteinyl leukotriene receptor $CysLT_1$ in the lungs and bronchial tubes by binding to it. This reduces the broncho constriction otherwise caused by the leukotriene and results in less inflammation. It is used for the treatment of bronchial asthma[5.]

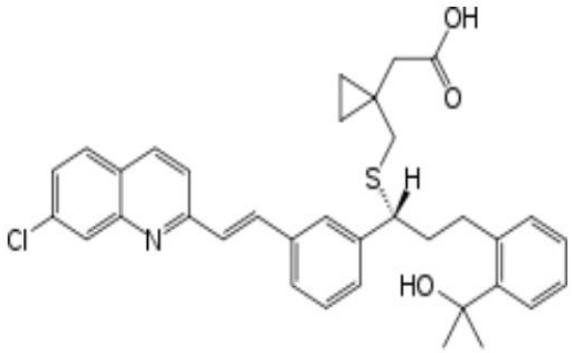

FIG 1: Structure of Montelukast

Based on the executed Literature review, it is noticed thatvery few methods was available for the "Development and Validation of a bio-analytical method for determination of Montelukast in human plasma byLC-MS/MS[6-8]." However, the current experimental study was proved to be more accuate and with lesser analytical time.

Thus, the aim of this study was to simplify sample preparation step using protein precipitation and simultaneously to shorten the chromatographic run time with a more selective LC-MS/MS procedure. Further, to improve the precision and accuracy of the method isotopically labeled Montelukast was used (Montelukast-D6) to reduce matrix effect and reproducibility. These improvements enabled development of a rapid, selective and sensitive LC-MS/MS method for determination of Montelukast in human plasma. It is important to develop the superior bio analytical method with proper deuterated or analogue based internal standards in terms of reduce matrix effect and improve. Reproducibility[9,10.]

The present study describes, the development and validation of an isocratic LC-MS/MS with highly efficient, more specific and highly sensitive, simple extraction, good linear method for quantitative determination of Montelukast in human plasma with the small amount of plasma usage as per bio analytical ICH M10 guideline[11-12]

EXPERIMENTAL

Materials and Methods

Materials

Montelukast, Montelukast D6 sodium salt was gifted by Spectrum Labs , Hyderabad.

Human plasma

K_2 EDTA control plasma procured by Deccan Pathological labs, Hyderabad.

Chemicals and solvents

Montelukast reference standard Montelukast D6 reference standard Orpheus C_{18} 100mg/1.0mL cartridgesAcetonitrile (HPLC grade)

Methanol(HPLCgrade) Milli-Q water Ammonium acetate(ARgrade)Formic acid (GR grade) Human plasma 0.45µ Membrane filter

TABLE 1: List of Instruments

HPLC System	Shimadzu
Mass Spectrometer	API 3000, MDS Sciex
Deep Freezer	Sanyo (-86°C) VIP Series
Microbalance	Sartorius
Vibramax	Heidolph
Vacuum pump	Millipore
Refrigerator	Samsung
PH meter	Orion
Micropipettes, Multipette and Micro tips	Brand and Eppendorf
Vortexer	Spinix
Solid phase extraction chamber	Orochem
Orpheus C_{18} 100mg/1.0mL cartridges	Orochem
Poly propylene tubes	Torson's
Water Purification System	Elix 10 / Milli-Q gradient
Ultra sonicator	Bandelinsonorex
Nitrogen Evaporator	ZymarkTurbovap LV station, Caliper

Preparation of solutions

Montelukast Stock Solution

Weighed accurately, about 5 mg of Montelukast Working standard separately and transferred to a separate 5 mL clean glass volumetric flask, dissolved HPLC grade methanol and made up the volume with the same to produce a solution of1000000.00 ng/mL. Corrected the above concentration of Montelukast solution accounting for itspotency and the actual amount weighed for free compound weight. A batch number was provided and the 'Stock Weighing and Solution Preparation' form was completed. The stock solution was stored in refrigerator at 2-8°C and used for maximum of four days.

The stock solution was diluted to suitable concentrations using diluents for spiking in to plasma to obtain calibration curve (CC) standards, quality control (QC) samples and DIQC samples. All other dilutions (system suitability dilutions, aqueous mixture, recovery etc.) were prepared in mobile phase.

Montelukast D6 Stock Solution (Internal Standard)

Weighed accurately, about 5.0000 mg of Montelukast D_6 sodium and transferred to a separate 5mL volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same to produce a solution of 1000000.0000 ng/mL. Corrected the above concentration of Montelukast D_6 accounting for its potency and the actual amount weighed. A batch number was provided and the 'Stock Weighing and Solution Preparation' form was completed. The stock solution was stored in refrigerator at 2-8°C and used for maximum of four days. The stock solution was diluted to suitable concentration using diluents for internal standard dilution..

Calibration curve standards and quality control samples

Calibration curve standards consisting of a set of nine non-zero concentrations ranging from 5.032 ng/mL to 602.362 ng/mL for Montelukast were prepared. Quality control samples consisted of Montelukast concentrations of 5.036ng/mL (LLOQ QC),15.076 ng/mL (LQC), 90.278ng/mL (MQC1), 300.927ng/mL (MQC2) and 50.8885ng/mL (HQC) were prepared. These samples were stored below -70 °C until use. Twelve sets of LQC and HQC were transferred to the -20 °C deep freezer to check stability at -20 °C.

Standard	Concentration	Montelukast (ng/mL)
Standard I	2-3 times of Cmax	60.1740
Standard H	80% of I	48.0189
Standard G	60% of I	36.0141
Standard F	40% of I	24.0214
Standard E	20% of I	12.0107
Standard D	10% of I	6.0054
Standard C	5% of I	2.1019
Standard B	40% of C conc.	0.6095
Standard A	50% of B conc.	0.3048
LLOQ QC	Conc equal to A	5.036
LQC	2.5-3 times of LLOQ	15.076
MQC 1	50% of I	90.278
MQC 2	50% of I	300.927
HQC	75-90% OF I	50.8885

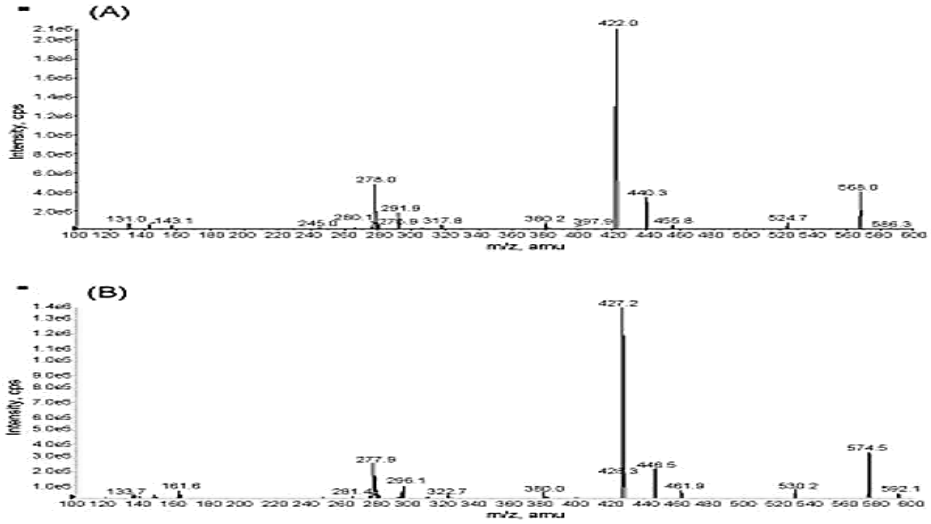

FIG 2: The mass spectrum of the drug molecule is given

Retention times of Montelukast is 1.70 ± 0.3 min &Montelukast D6 is 1.70 ± 0.3 min.

Detection of Montelukast Parent mass (amu) is 586.30 & product mass is 422.20

Detection of Montelukast D6 Parent mass (amu) is 592.40 & product mass is 427.10

Optimization of the chromatographic conditions

The reconstituted samples were chromatographed on a ZORBAX Eclipse XDB Phenyl (4.6 X 75 mm, 3.5µ) by using Acetonitrile: 5mM Ammonium acetate buffer (85:15 v/v) as the mobile phase at a flow rate of 0.6.0 mL/min.Detection was carried out LC-MS/MS (API 3000) in negative ion

Parameter	Value
Column	AX Eclipse XDB Phenyl(4.6 X 75 mm, 3.5µ)
Mobile phase	Acetonitrile: 5mM ammonium acetate buffer (85:15 v/v)
Buffer	5mM ammonium acetate buffer

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Isocratic/gradient mode	Isocratic
Flow rate	0.60 mL/min
Run time	3.0 min
Column oven temperature	40 ± 2^0C
Auto sampler temperature	5^0C
Volume of injection	20 µL
Rinsing volume	700 µL

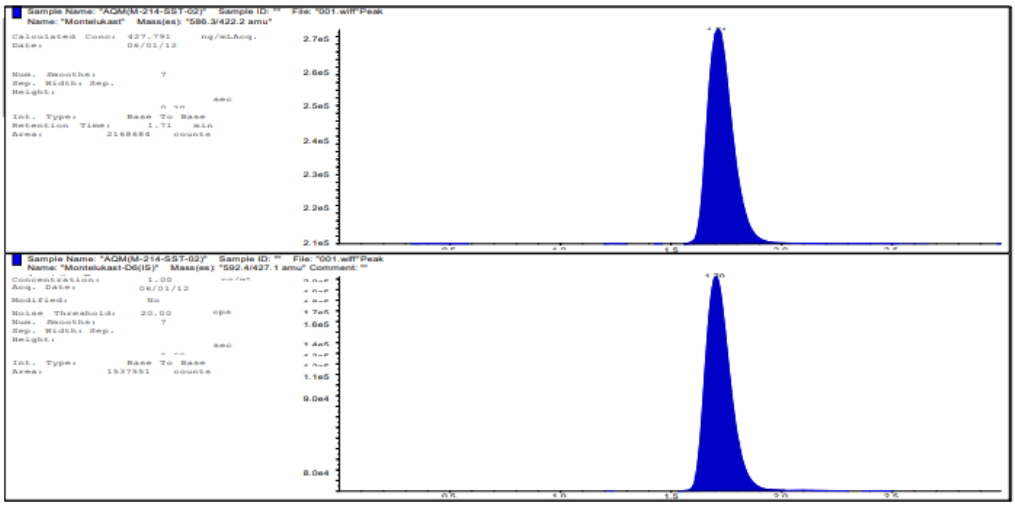

FIG 3: Representative Chromatogram of an Aqueous Standard and InternalStandard of Mixture of Montelukast

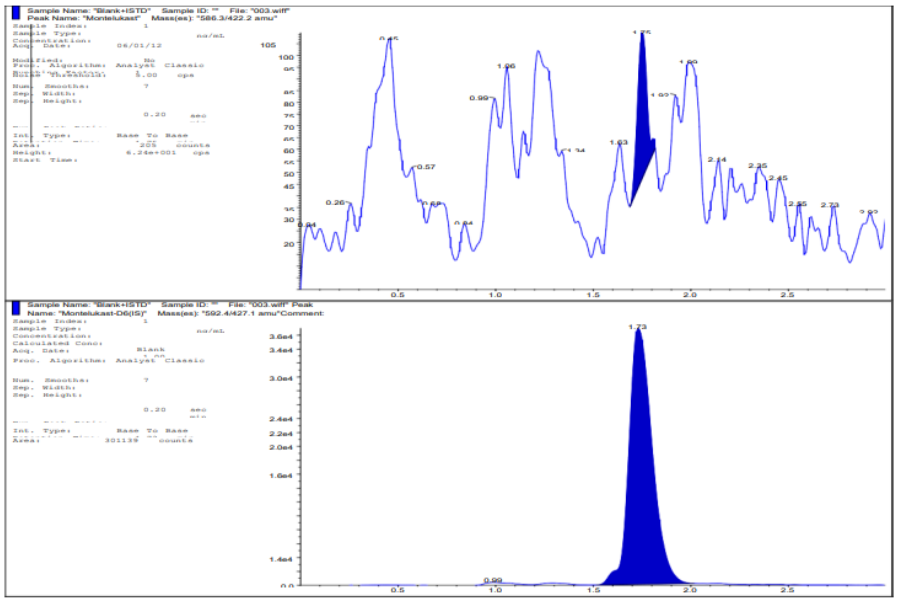

FIG 4: A Representative Chromatogram of Blank Plasma with Internal StandardSample of Montelukast

Validation

ICH M10 guidelines were followed formethod validation the method was validated for its selectivity, stability, linearity, accuracy, precision, and recovery.

Selectivity

The selectivity of the method was assessed by comparing chromatogram of blank plasma and spiked plasma. The retention times were 1.70 min for analyte and 1.70 for internal standard

represented in Figs. (**2**, **3**). There were no significant endogenous peaks. That could not interfere with retention time of Analyte and Internal standard. The results indicate that the method exhibited good selectivity.

% Interference of Analyte and IS=Mean Interference at RT of Analytes and ISX 100

Average response in Selectivity LLOQ

Sensitivity

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 97.04%. It can be concluded that the sensitivity is more for this method.

Matrix effect

Matrix effect for Montelukast was evaluated by analyzing all the eight batches of plasma at low (LQC) and high (HQC) concentrations. No significant matrix effect was observed in all the eight batches of plasma for Montelukast at low (LQC) and high (HQC) concentrations. The precision for IS normalized matrix factor at LQC and HQC level was found to be 0.69% and 0.61%, respectively. The results were within the acceptable limits and given in tables 5 & 6.

Linearity

The linearity of the method was determined by a weighted ($1/X^2$ where X is concentration) least square regression analysis of the standard plots associated with the eight point standard curve for Montelukast. The calibration line was linear in the range of 5.032ng/mL to 602.362ng/mL of the drug as shown in Fig:11. A straight-line fit made through the data points by least square regression analysis showed a constantproportionality with minimal data scattering. The correlation coefficient (r^2) was greater than 0.99 and ranged from 0.9960 to 0.9972 for Montelukast.

TABLE 2: Concentration-response Linearity Data of Montelukast

Montelukast	**Concentration (ng/mL)**									Slope	Intercept	r^2
	STD-A	STD-B	STD-C	STD-D	STD-E	STD-F	STD-G	STD-H	STD-I			
Upper Limit	6.038	11.574	34.653	69.306	138.613	277.225	415.630	554.172	692.716			
Lower Limit	4.026	8.554	25.613	51.226	102.453	204.905	307.204	409.606	512.008			
CC	5.032	10.064	30.133	60.266	120.533	241.065	361.417	481.889	602.362			
1	4.893	10.480	30.959	61.758	125.181	245.090	319.380	484.142	596.639	0.0033	0.0005	0.9970
2	4.910	10.371	31.010	63.903	122.137	244.669	314.973	474.404	614.448	0.0035	0.0007	0.9964
3	5.172	9.285	31.283	63.883	123.255	237.336	323.255	485.843	627.532	0.0019	-0.0002	0.9960
4	4.947	10.333	30.013	64.600	117.708	244.903	324.430	485.006	617.807	0.0033	-0.0018	0.9972
Mean	4.9805	10.1173	30.8163	63.5360	122.0703	242.9995	320.5095	482.3488	614.1065			
SD	0.12964	0.55832	0.55407	1.23132	3.16828	3.77959	4.27538	5.34183	12.89920			
CV%	2.60	5.52	1.80	1.94	2.60	1.56	1.33	1.11	2.10			
% Nominal	98.98	100.53	102.27	105.43	101.28	100.80	88.68	100.10	101.95			
N	4	4	4	4	4	4	4	4	4			

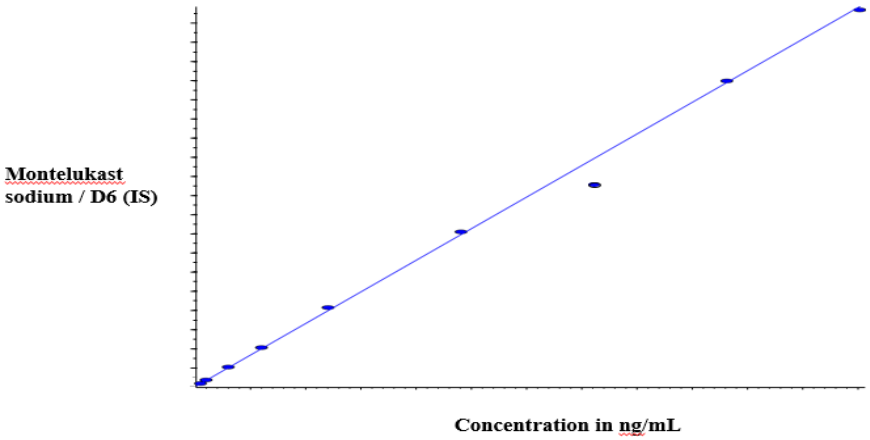

FIG 11: Representative Calibration Curve for Regression Analysis of Montel

Precision and Accuracy

The precision was less than 4.67% and the accuracy of the mean of measured concentrations ranged from 97.48 to 106.68%. Precision and accuracy for this method were controlled by calculating the intra and inter-batch variations of QC samples in six replicates. The intra-batch precision and accuracy were between 4.67 to 6.24 and 100.85 to 112.38%. Similarly, the inter-batch precision and accuracywere between 4.69 to 6.69 and 102.26 to 107.17% are summarized in Table 3. These results indicate the adequate reliability and reproducibility of this method within the analytical range.

Recovery

The overall average recoveries of analyte and IS were found to be 58.56%and57.75%. Recoveries of the analyte and IS were high and consistent, precise and reproducible.

Stability

Analyte in plasma was subjected to three freeze/thawcycles. The obtained accuracy was between 102.26% and 107.17% of the theoretical values. No significant degradation of the analyte was observed even after 48 h storage period in Autosampler tray and the final concentrations of analyte was found in between 91.96% and 109.15% of the theoretical values. In addition, the long-term stability of QC samples after 90 days of storage was at -20^0c, -50^0c. The concentrations ranged from 92.24 to 95.13% for long term stability and room temperature stability for 48 h was also evaluated for Analyte and IS. The % comparison response 101.96 to 93.88% for Room Temperature and Refrigeration stock solution stability studies. These results confirmed the stability of analyte human plasma for at least 90 Days at -20^0c,

Reinjection Reproducibility

In accessing the reinjection stability, six sets of QC samples (LQC and HQC) were processed and analyzed with calibration curve standard. The QC samples were retained in the autosampler and reinjected after a period of 44 hours and quantified against the initial calibration curve data, refer, Table 11. 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.

SUMMARY OF RESULTS

VALIDATION PARAMETER		Montelukast		Montelukast D_6	
		% Nominal / %Stability	Precision	% Nominal / % Stability	Precision
Biological Matrix		Plasma	N/AP	Plasma	N/AP
Detection		m/z – 586.30 (parent) and 422.20 (product)		(parent) and 427.10(product)	
Analytical Range		5.032 ng/mL – 602.362 ng/mL		N/AP	N/AP
Minimum Quantifiable Concentration		5.032 ng/mL		N/AP	N/AP
Matrix Effect	Normalized Matrix Factor at LQC	0.69%		N/AP	N/AP
	Normalized Matrix Factor at HQC	0.61%		N/AP	N/AP
Sensitivity		97.04%	3.10%	N/AP	N/AP
Coefficient of correlation (r^2)		0.9960-0.9972		N/AP	N/AP
Within Batch Precision and Accuracy		.48%-106.68%(LLOQ QC), 104.66%-109.12%(LQC) 98.80%-112.98%(MQC1) 96.97%-110.36% (MQC2) 88.73%-112.06% (HQC)	.34%-7.62%(LLOQ QC) 1.08%-7.47% (LQC) 2.27%-6.32% (MQC1) 1.80%-6.05% MQC2) 0.93%-3.98%(HQC)	N/AP	N/AP
Intra Day Precision and Accuracy		100.85%(LLOQ QC) 106.89%(LQC) 112.38%(MQC1) 110.33% (MQC2) 108.22% (HQC)	6.90%(LLOQ QC) 6.24%(LQC) 4.75%(MQC1) 4.84%(MQC2) 4.67%(HQC)	N/AP	N/AP
Between Batch / Inter Day Precision and Accuracy		103.40%(LLOQ QC) 106.82%(LQC) 107.17%(MQC1) 106.55% (MQC2) 102.26% (HQC)	6.69%(LLOQ QC) 4.69%(LQC) 6.64%(MQC1) 6.58%(MQC2) 8.78%(HQC)	N/AP	N/AP
Re Injection Stability (44 hrs)		92.86% to 97.13%	0.76% to 2.94%	N/AP	N/AP
Room Temperature Montelukast Stock Solution Stability (6 hrs)		101.96%	0.56% - 0.73%	N/AP	N/AP
Room Temperature IS Stock Solution Stability (7 hrs)		N/AP	N/AP	102.33	0.48% to 1.04%
Room Temperature Spiking Solution Stability (7 hrs)		102.77%	0.30% - 0.56%	102.67%	0.48%-0.69%
Refrigerated Stock Solution Stability(4 days)		93.88%	2.06% - 2.59%	93.28%	2.43% - 3.17%
Auto Sampler Stability (51 hrs)		91.96% - 109.15%	0.95% - 1.86%	N/AP	N/AP
Freeze Thaw Stability (4 Cycle)		90.64% - 107.39%	1.31% -1.15%	N/AP	N/AP
Bench Top Stability (10 hours)		90.34% - 107.21%	0.68% - 2.06%	N/AP	N/AP
Short term –20°CStability (4 days)		98.59% - 102.14%	1.66%-1.76%	N/AP	N/AP
Wet Extract Stability (50 hrs)		90.93% -107.42%	0.88% - 1.72%.		

Recovery	58.56%	1.00% to 5.17%	57.75%	4.25% to 5.08%
Dilution Integrity: Two times dilution	95.03%	0.72%	N/AP	N/AP
Dilution Integrity: Four times dilution	97.97%	0.98%	N/AP	N/AP
Precision and Accuracy(Ruggedness)	106.68%(LLOQ QC) 108.35%(LQC) 105.14%(MQC1) 108.55% (MQC2) 103.87% (HQC)	5.50%(LLOQ QC) 1.08%(LQC) 2.27%(MQC1) 1.80%(MQC2) 1.81%(HQC)	N/AP	N/AP

CONCLUSION

Thus, the objective was to develop and validate suitable method for estimation of unknown concentration of drug in plasma. A highly accurate, sensitive, specific and reproducible LC-MS/MS method for the quantification of Lansoprazole using commercially available IS from small volumes of human plasma with a simple Solid Phase Extraction process was developed and validated. Developed and validated analytical method was found to be simple rapid, specific, sensitive, precise and cost effective than the other reported methods. The method has been successfully applied to the investigation of a preclinical pharmacokinetic study with desired precision and accuracy along with high throughput.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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