Development and Validation of LC-ESI-MS/MS Based Bioanalytical Method for Ripretinib in Rat Plasma and its Applications to Pharmacokinetic Studies

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Abstract-The present study aimed at development and validation of a simple, rapid, sensitive and specific reverse phase liquid chromatography-electro spray ionization tandem mass spectrometry method for quantification of Ripretinib in bulk and pharmaceutical dosage form in rat plasma using D6-Ripretinib as the internal standard and pharmacokinetic properties evaluation. Wavelength at which strongest photon absorption seen is 220nm using photodiode array detector.Liquid-liquid extraction technique followed for extraction of Ripretinib from rat plasma. Detection was carried out using MRM with positive ion mode at transitions of 578.45m/z→197.32m/z for Ripretinib and 516.89m/z→436.57m/z for D6-ripretinib.The chromatographic separation of Ripretinib is achieved using, waters alliance high performance liquid chromatography, isocratic pump of e2695, Waters X-Bridge C18 150mmx4.6,3.5µm column, mobile phase containing acetonitrile and buffer 0.1%trifluroacetic acid in the ratio of 50:50%v/v with injection volume of 5µl at a flow rate of 0.5ml/min and run time of 4mins.The peak elution of Ripretinib was observed at the retention time of 2. 887min.The calibration curve was linear over the range 88-700ng/ml with a regression coefficient(r^2) 0.999, %relative standard deviation of peak areas was found to be <2.0 which is in accordance with the acceptance criteria.The developed method was validated according to the USFDA guidelines and all the validation parameters are found to be within the limits.Stability studies were performed and found to be stable under the analytical conditions used.The pharmacokinetic study observations of orally administered tablet dosage form in rats were C_{max}320.1ng/ml at a $t_{max}$of 4hrs.

Keywords: Ripretinib, Plasma, Pharmacokinetic, liquidliquid extraction, validation.

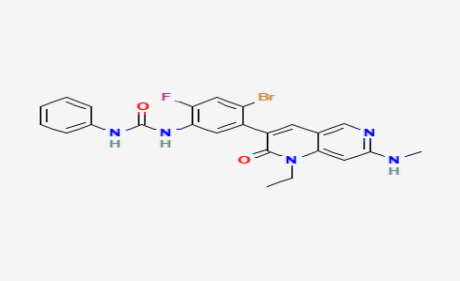

Fig.1Structure of Ripretinib

INTRODUCTION

IUPAC name of Ripretinib1-{4-bromo-5-[1-ethyl-7-(methylamino)-2-oxo-1,2-dihydro-1,6-naphthyridin-3-yl]-2-fluorophenyl}-3-phenylurea.Molecular formula and molecular weight of Ripretinib given as$C_{31}H_{34}F_2N_6O_2$ and 510.36 g/mol respectively [1].Ripretinib (brand nameQin lock).Approved by FDA on May 15, 2020[2].Ripretinib is used to treat adults diagnosed with advanced gastrointestinal stromal tumour (GIST) who had previously undergone therapy with at least 3 kinase inhibitors, including the imatinib [3].Majority of gastrointestinal stromal tumour (GIST) are caused due to protein kinases dysregulation that led to carcinogenesis.Ripretinib inhibits protein kinases including wild type and mutant platelet-derived growth factor receptor A (PDGFRA)[4].Ripretinib by binding to the kinase switch pocket as well as the activation loop, turns off the kinase and its ability to cause dysregulated cell growth [5].The literature survey of the drug revealed that there were no analytical works carried out so far for quantification of Ripretinib so, LC-MS/MS method was selected over the methods that are available since it gives information about the structure, molecular formula of the compound and determination of desired analyte from biological fluids.Pharmaceutical industries applying for investigational new drug (IND)application need to submit bioavailability and bioequivalence studies which require pharmacokinetic evaluation, toxicology studies, preclinical studies where bioanalytical method development and

validation plays a major role.The reason behind the investigation is to develop and validate a method to identify and quantify drug in biological matrix (rat plasma) and analysing pharmacokinetic properties. During the literature survey the molecular docking study indicated that Ripretinib strongly docks with three known novel severe acute respiratory syndrome coronavirus 2 (SARS-n-CoV-2) proteins with a reasonably good docking score.

MATERIALS AND METHODS

Ripretinib (standard and formulation), D6-Ripretinib (internal standard) were purchased from ZydusCadila, Ahmedabad, India.Acetonitrile LCMS grade and Trifluoroacetic acid LCMS grade were obtained from Merck, Mumbai, Water was from In-house production and six different lots of rat plasma from local suppliers.

Instrumentation

Waters, alliance e-2695 model HPLC system with photodiode array detector (PDA) was coupled to SCIEX QTRAP 5500 mass spectrometer equipped with electrospray ionization interface.Chromatographic data processed using Empower-2 software.HPLC system was provided with column oven, auto sampler and degasser for analysis.Endeavour analytical balance used for weighing purpose.Grant ultrasonic bath used for mixing and dissolving of analyte.Cyclomixer for proper mixing of contents in centrifuge tube.Remi laboratory centrifuge for separation of clear liquid.Glass vacuum filtration unit for filtration of buffer.pHmeter for determining p^H of buffer.

Optimized liquid chromatography and mass spectroscopic parameters:

Optimized liquid chromatographic conditions shown in table1

Column name	Waters X-Bridge
Type	C18
Dimensions	150×4.6,3.5µm
Buffer p^H	0.1%Trifluoroacetic acid,2.3
Mobile phase	Acetonitrile:0.1%Trifluoroaceticacid(50:50%v/v)
Pump mode	Isocratic
Flow rate	0.5ml/min
Injection volume	5µl
Run time	4mins
Wavelength	220nm
Retention time (R_t)	Ripretinib-2.887mins D6-Ripretinib-2.885mins

optimized mass spectroscopic conditions shown in table2

MS	Sciex QTRAP 5500	Ionization type	Electro spray ionization (ESI)
	Drying gas: N_2 gas	Collision cell gas	Nitrogen with high purity
	Drying flow rate: 5 ml/min	Mode	MRM, positive ion
	Drying gas temperature: 120-250°C	Ripretinib MRM transitions	m/z-578.45→m/z-197.32 CE[a]– 15V
	Pressure: 55 psi		
Ionization source	Source temperature: 550°C	D_6Ripretinib MRM transitions	m/z-516.89→ m/z-436.57 CE[a] - 13V
	Capillary ion spray voltage: 5500V Declustering potential: 40 V Entrance potential: 10V exit Potential: 7 V Dwell time: 1sec		

[a]CE-Collision energy, [b]MRM- Multi reaction monitoring transition

Preparation of standards and quality control samples:

Standard stock solution of Ripretinib and internal standard(D6-ripretinib) were prepared by taking 5mg in a mixture ofacetonitrile and water (50:50%v/v) at a concentration of 1000µg/ml.The stock solution of Ripretinib and internal standard stock was serially diluted with acetonitrile:0.1%trifluoroacetic acid(50:50%v/v) to provide working standard solution of desired concentration.Calibration standards were prepared by spiking of blank plasma with Ripretinib working standard solutions.The effective concentration in standard plasma samples were 87.5,175,262.5,350,437.5,525,700ng/ml

each containing 350ng/ml of D6-ripretinib solution as an internal standard. Four levels of quality control samples (QCs) prepared with plasma at LLQC (35ng/ml), LQC (175ng/ml), MQC (350ng/ml) and HQC (525ng/ml).

Accuracy sample stock and lower limit quality control sample stock prepared by taking 5mg Ripretiniband 7mg Hydroxy propyl methylcellulose in a mixture of acetonitrile and 0.1%trifluoroace tic acid (50:50%v/v), this is further diluted to provide working standard of desired concentration.

Preparation of sample stock:

Formulation sample stock prepared by taking tablet powder which is equivalent to 12mg (5mg drug and 7mg excipients from tablet strip of label claim 50mg and average weight of tablet 120mg which is grinded by means of motor and a pestle, in a mixture of acetonitrile and 0.1%trifluoroacetic acid (50:50%v/v), this is further diluted to provide working standard of desired concentration.

Sample extraction procedure:

Liquid-liquid extraction was performed to extract drug and internal standard.Plasma samples are withdrawn from the deep freezer and thawed to room temperature.Calibration curve standards of different concentrations and quality control samples are prepared by taking, initially 200µl each of rat plasma and acetonitrile into centrifuge tubes and capped and placed on the cyclomixer and vortexed for 3mins for proper mixing and precipitation of proteins.Later different volumes of working standard stock of Ripretinib are taken into centrifuge tubes and 100µl of D6-Ripretinib is taken into each tube using micropipette and final volume was adjusted to 2000µl(2ml) with mixture of acetonitrile:0.1%trifluoroacetic acid(50:50%v/v), tubes are capped and again placed on cyclomixer and vortexed for 3mins for proper mixing of contents.These tubes are placed in centrifuge and centrifuged at a speed of 1100rpm for 15mins, then clear supernatant top layer was collected by means of syringe and filtered through nylon filter of 0.45µ filter and the obtained filtrate was collected in a respectively labelled auto injection vial.

Data processing:

The chromatographic data processed using Empower 2software.SCIEX software was used for the interpretation of the data of the chromatogram.

RESULTS

The developed method was validated to persuade the acceptance criteria of Industrial guidance for the bioanalytical method validation USFDA guidelines.During the method development stage several trails were carried out, better elution of the peaks was observed with mobile phase containing acetonitrile and 0.1%trifluroacetic acid in the ratio of(50:50%v/v) and Waters X-Bridge C18 150mmx4.6,3.5µm column and retention times of Ripretinib and D6-Ripretinib were found to be 2.887mins and 2.885mins shown in fig 2,3 respectively due to ready elution of peaks the developed method was found to be economical as mobile phase required is less.

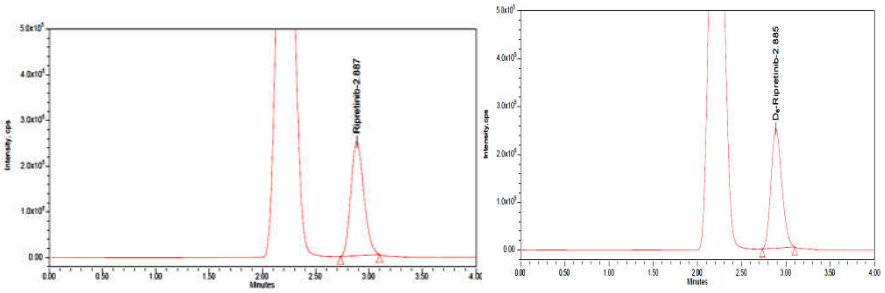

Fig.2: Chromatogram of RipretinibFig.3: Chromatogram of D6-Ripretinb

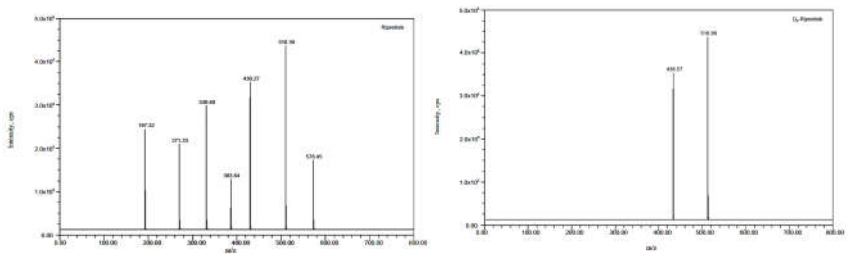

Fig.4: MS Spectra of RipretinibFig.5: MS Spectra of D6-Ripretinib

Linearity:

Linearity was performed by assaying plasma samples at 7 different concentrationsover the concentration range of 88-700ng/ml of Ripretinib.The standard curve was plotted by taking concentration of Ripretinib on x-axis againstpeak area response of Ripretinib to internal standard on y-axis. Regression coefficient (r^2) is determined from the graph.

The calibration curve was found to be linear over the concentration range of 88-700ng/ml with a regression coefficient of 0.999 shown in fig 6 and results are shown in table3.

TABLE 3:RESULTS OF LINEARITY

Linearity	Ripretinib conc(ng/ml)	Ripretinib response	Internal standard response	Area response ratio
Linearity-1	88	0.642	2.550	0.252
Linearity-2	175	1.333	2.580	0.517
Linearity-3	263	1.986	2.548	0.779
Linearity-4	350	2.591	2.532	1.023
Linearity-5	438	3.174	2.564	1.238
Linearity-6	525	3.756	2.597	1.446
Linearity-7	700	5.057	2.570	1.968
Slope Intercept R^2	0.0028 0.0142 0.999			

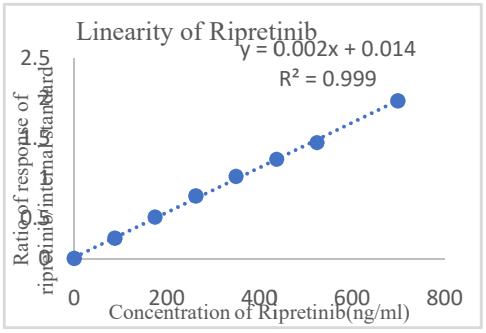

Fig.6:Calibration plot for concentration v/s

Area ratio of Ripretinib

LOD and LOQ

LODand LOQ were separately determined by the calibration curve method.LOD and LOQ of the compound were determined by injecting progressively lowerconcentrations of standard solutions using the developed method.The LOD and LOQ concentrations and s/n values are shown in table4

TABLE4: LOD and LOQ data for Ripretinib

Name	LOD		LOQ	
	Concentration(ng/ml)	s/n	Concentration(ng/ml)	s/n
Ripretinib	10.5	4	35	24

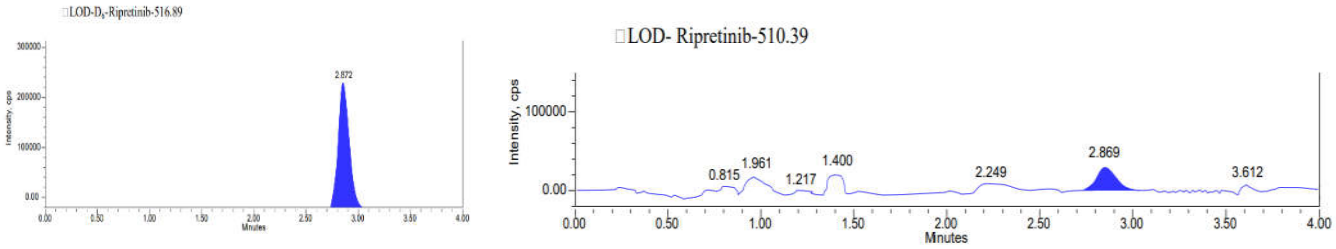

Fig.7: LOD peaks of Ripretinib

Specificity and selectivity

This test was performed by analysing the blank plasmasamples from six different lots to detectforany chromatographic interference at the retention times of the Ripretinib and Internal standard.The developed method was found to be highly specific as no interference observed at the retention time of Ripretinib and internal standard shown in fig8,9,10.

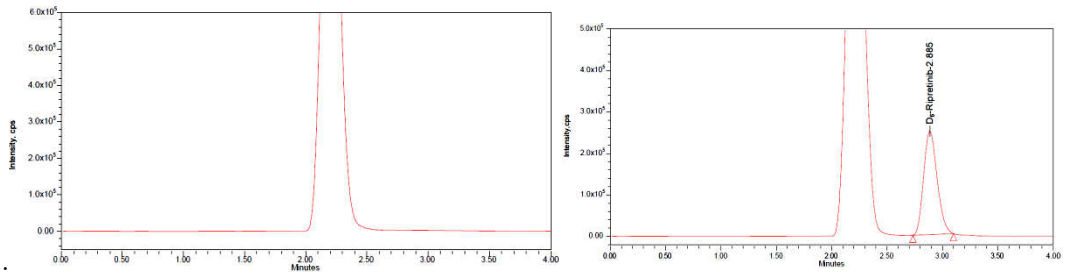

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Fig.8: Chromatogram of Blank PlasmaFig.9: Blank plasma spiked with IS

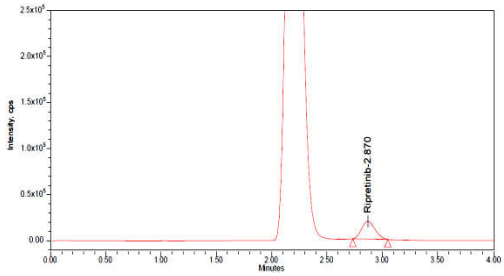

Fig.10: Blank plasma spiked with analyte

System suitability

It is used to indicate whether the instrument under current use is working properly or not and serves as an indication to proceed with the assaying of the next batch of samples. System suitability checks are more used for chromatographic methods to ensure that the system is sufficiently sensitive, specific and reproducible for the current analytical run throughout its use during a particular study. System suitability samples were included at the beginning, middle and end of each batch of samples. The final concentration of the system suitability samples was made up to contain 350ng/ml Ripretinib, 350ng/mlInternal standard in mobile phase.

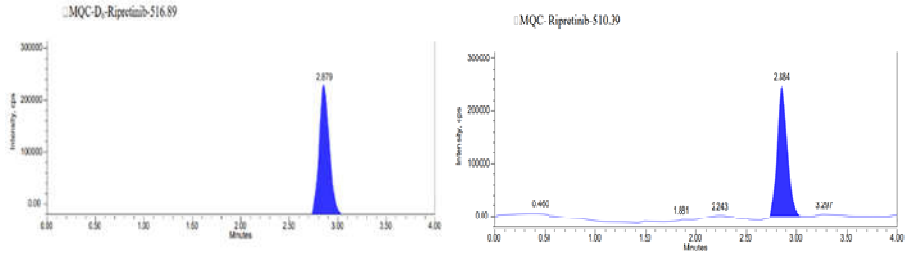

Fig.11: system suitability graphs

Precision and accuracy

The method precision and accuracy were evaluated by analyzing six replicates containing Ripretinib at four concentrations of LLQC, LQC, MQC and HQC.Intra-day evaluation was done on the same day, whereas inter-day was done on three consecutive days, performed by different analysts in different labs.The mean concentration, standard deviation (SD) and coefficient of variation (%CV) were evaluated.The developed method was found to be accurate and precise as the values fall in acceptance criteria in USFDA guidelines.The intra -day accuracy and precision of Ripretinib is found to be 91.58-106.37% and 0.08-0.82%.The inter-day accuracy and precision of Ripretinib is found to be 88.11-104.01% and 0.15-0.39% shown in table 5.

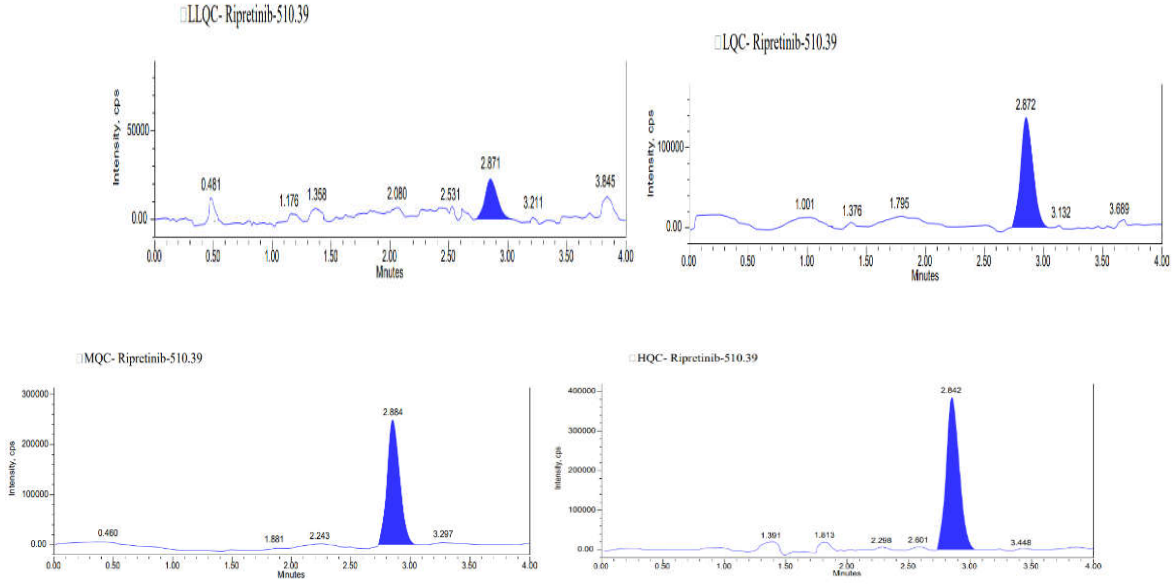

Fig.12: graphs for Ripretinib at LLOQ, LQC, MQC, HQC

TABLE5: INTRA DAY AND INTER DAY ACCURACY AND PRECISION(n=6)

Quality control sample	Spiked concentration (ng/ml)	Mean (ng/ml)	SD	Accuracy (%)	RSD (%)
Intra day					
LLOQ	$0.2351x10^5$	0.2352	0.0013	91.58	0.82
LQC	$1.3654x10^5$	1.3674	0.0244	106.37	0.16
MQC	$2.5729x10^5$	2.5729	0.0987	100.22	0.33
HQC	$3.8461x10^5$	3.8421	0.0375	99.88	0.08
Inter-day					
LLOQ	$0.2262x10^5$	0.2234	0.0049	88.11	0.39
LQC	$1.3351x10^5$	1.3318	0.0315	104.01	0.27
MQC	$2.5487x10^5$	2.5435	0.0632	99.28	0.18
HQC	$3.8263x10^5$	3.8254	0.0174	99.36	0.15

Recovery:

Recovery analysis was done by using six replicates at three different concentrations (LQC, MQC, HQC).The recovery of Ripretinib is obtained by comparing peak area obtained for plasma samples spiked with Ripretinib with that of peak area obtained for standard solution at equivalent concentrations.Spiked compounds resulted in recoveries of analyte 92.61 % - 114.85% shown in table6

TABLE6: RECOVERY OF ANALYTE OF RIPRETINIB

Replicate number	HQC		MQC		LQC	
	Extracted response	Unextracted response	Extracted response	Unextracted response	Extracted response	Unextracted response
1	$3.362x10^5$	$3.969x10^5$	$2.415x10^5$	$2.858x10^5$	$1.215 x10^5$	$1.761x10^5$
2	$3.365x10^5$	$3.945x10^5$	$2.458x10^5$	$2.825x10^5$	$1.214x10^5$	$1.787x10^5$
3	$3.425x10^5$	$3.925x10^5$	$2.369x10^5$	$2.847x10^5$	$1.253x10^5$	$1.723x10^5$
4	3.345_x10^5	$3.945x10^5$	$2.487x10^5$	$2.869x10^5$	$1.263x10^5$	$1.741x10^5$
5	$3.432x10^5$	$3.969x10^5$	$2.391x10^5$	$2.873x10^5$	$1.259x10^5$	$1.721x10^5$
6	$3.397x10^5$	$3.981x10^5$	$2.462x10^5$	$2.883x10^5$	$1.254x10^5$	$1.743x10^5$
Mean	$3.387x10^5$	$3.955x10^5$	$2.430x10^5$	$2.859x10^5$	$1.243x10^5$	$1.746x10^5$
SD	0.0359	0.0208	0.0458	0.0208	0.0223	0.0248
%RSD	1.05	0.52	1.88	0.72	1.79	1.42
%Mean Recovery	87.95%	102.70%	94.65%	111.37%	96.84%	112.02%
Mean %	98.99%					
Mean SD	1.1604					
Mean %RSD	0.43					

Matrix effect:

Matrix effect is determined by matrix factor which is affected by the components of biological matrix. Matrix factor equal to 1 indicates no suppression, matrix factor<1 indicates ion suppression effect, matrix factor>1 indicates enhancement.The matrix of plasma constituents over the ionization of analyte was determined by comparing the response ofpost-extracted plasma standard QC samples (n = 6) (3LQC's,3HQC's) of 6 different lots of plasma with the response of analyte from neat samples at equivalent concentrations. Matrix effect shown in table7.

TABLE7: MATRIX EFFECT

S.No.	Plasma Lot No.	HQC	LQC
		Nominal	Concentration(ng/ml)
		525.4733	175.5097
		Nominal Range	Concentration(ng/ml)
		(525.298-525.645)	(175.206-175.734)
		Calculated	Concentration(ng/ml)

1.	Lot 1	$3.8654x10^5$	$1.3785 x10^5$
		$3.8647x10^5$	$1.3754 x10^5$
		$3.8542x10^5$	$1.3741 x10^5$
2.	Lot 2	$3.8561x10^5$	$1.3847 x10^5$
		$3.8647x10^5$	$1.3741 x10^5$
		$3.8478x10^5$	$1.3784 x10^5$
3.	Lot 3	$3.8645x10^5$	$1.3743 x10^5$
		$3.8678x10^5$	$1.3764 x10^5$
		$3.8621x10^5$	$1.3747 x10^5$
4.	Lot 4	$3.8610x10^5$	$1.3786 x10^5$
		$3.8591x10^5$	$1.3738 x10^5$
		$3.8612x10^5$	$1.3771 x10^5$
5.	Lot 5	$3.8691x10^5$	$1.3781 x10^5$
		$3.8617 x10^5$	$1.3794 x10^5$
		$3.8643x10^5$	$1.3763 x10^5$
6.	Lot 6	$3.8694 x10^5$	$1.3791 x10^5$
		$3.8678x10^5$	$1.3781 x10^5$
		$3.8649x10^5$	$1.3722 x10^5$
N	18	18	
Mean	3.8625	1.3769	
SD	0.00553	0.00291	
%CV	0.14	0.21	
% Mean Accuracy	100.29%	107.2%	
No. of QC Failed	0	0	

STABILITY STUDIES

Bench top stability:

For bench top stability experiment, stability ofRipretinib. in the rat plasma after 8 h exposure to room temperature was determined at three concentrations (LQC, MQC and HQC) in sixreplicates.

Freeze-thaw stability

Six replicates of each (LQC, MQC and HQC) that were stored at - 20 ^{O}C, were thawed completely thawing at room temperature and refrozen immediately to -20 ^{O}C.This cycle wasrepeated twice and the samples were extracted for injection in toLCMS.

Wet Extract stability

Freezer stability of Ripretinib in plasma was assessed by analysing LQC, MQC and HQC samples in six replicates stored at -20 ° for 24 Hours for stability study.All sample compared with the fresh prepare samples of three different QC in six replicates.

Auto sampler Stability

Samples of Ripretinib in plasma was assessed by analysing LQC, MQC and HQC samples are injected every 1 hour up to 24 Hours for stability study.All sample compared with the fresh prepare samples of 0Hr of different QC in six replicates.

Dry extract stability

Dry extract stability of Ripretinib in plasma was assessed by analysing LQC, MQC and HQC samples in six replicates stored at -20 °C for 12hrs, after completion of 12hrs stored at -20 for 12hrs, after completion of 24hrs injected into LCMS system.

Short term stability

Short term stability of Ripretinib in plasma was assessed by analysing LQC, MQC and HQC samples in six replicates stored at 5° for one week and injected into LCMS system.

Long term stability

Long term stability of Ripretinib in plasma was assessed by analysing LQC, MQC and HQC samples in six replicates stored at -20° for 4 weeks and each week the stability is assessed.

TABLE8: STABILITY STUDIES

Stability	Storage condition	Conc level(ng/ml)	%RSD	%Recovery
Bench top stability	24 h at room temperature	LQC	1.22	104.46
		HQC	0.30	99.73
Autosampler stability	24 h in auto sampler at room temperature	LQC	0.67	107.33
		MQC	0.29	100.22
		HQC	0.12	99.96
Long term stability (Day 28)	Day-1 at (-20±3) °C	LQC	1.74	104.63
		HQC	0.31	99.59
	Day-7 at (-20±3) °C	LQC	0.18	99.18
		HQC	0.05	97.73
	Day-14 at (-20±3) °C	LQC	0.20	96.15
		HQC	0.09	96.46
	Day-21 at (20±3)°C	LQC	0.15	92.23
		HQC	0.06	95.66
	Day-28 at(-20±3)°C	LQC	0.17	87.62
		HQC	0.06	94.11
Freeze thaw stability	24 h at (28±5) °C then exposed to three freeze and thaw cycles	LQC	1.66	105.55
		HQC	0.48	99.71
Wet extract stability	12 h at 2-8°C	LQC	0.36	106.01
		HQC	0.39	100.44
	18 h at 2-8°C	LQC	1.88	104.65
		HQC	0.69	99.9
Dry extract stability	12 h at (-20±3) °C	LQC	1.64	105.94
		HQC	0.57	100.22
	18 h at (-20±3) °C	LQC	1.43	104.52
		HQC	0.56	1.43
Short term stability	Day-7 at (5±3) °C	LQC	0.20	99.29

PHARMACOKINETIC STUDY

In this study three healthy white albino rats (body weight in between 250-350grams) were used.The protocol of animal study was approved by institute of animal ethics committee (Reg.No:1074/PO/Re/S/07/CPCSEA) and Animal Ethic Committee number for carrying out experimentation (ML/AR/021/125819).For investigating pharmacokinetic parameters of Ripretinib, market formulation dosage of 0.21 mg/1 kg Ripretinib was injected into rat body as intravenous administration.Blood sample volume of 1-2 mL was collected from the rat body in different time periods, like 0.5, 1, 2, 3, 4, 5, 6, 10, 15 and 20 hrs from the administered time of the drug into the rat body.After that 0.8 ml of each sample was diluted to 2 ml.The prepared samples were injected into the chromatographic system and record the values.The supporting data has been fortunate to quantify Ripretinib concentrations in six groups of rats under abstain from food state after administration of the drug as an oral dose.After that samples are prepared as per test method and injected into the LCMS/MS chromatographic systems and record the values.The pharmacokinetic characteristics estimated were C_{max} (highest seen drug accumulation during the steady), AUC_{0-12} (peak response below the vital fluid concentration, time loop measured, utilizing the rule of trapezoid), t_{max} (time to Max seen highest drogue accumulation), k_{el} (pictured terminal calculated constant of the first order rate from the half log graph of planetary plasma accumulation versus curve time, using the procedure regression of the least square) and t1 /2 (end half-way was calculated by using the blueprint formula 0.693 per k_{el}.The above optimised method was successfully applied to determine pharmacokinetic parameters shown in table 9 dosage calculation, table10,11 and fig9.

TABLE 9: DOSE CALCULATION FOR PHARMACOKINETIC STUDY

Subject	weight	LC
Human body	60kg	50mg
	1kg	0.83 mg
	1000mg	0.83 mg
Rat body	250mg	0.21 mg

TABLE10: PHARMACOKINETIC RESULTS OF RIPRETINIB

Time(hrs)	Average conc
0	0
0.5	46.02
1.0	99.98
2.0	182.77
3.0	264.37
4.0	320.11
5.0	250.57
6.0	191.22
10.0	90.80
15.0	34.18
20.0	0.00

TABLE 11: PHARMACOKINETIC PARAMETERS OF RIPRETINIB

Pharmacokinetic parameter	Ripretinib
AUC_{0-t} (ng h/ml)	2660
C_{max}(ng/ml)	320.1
$AUC_{0-\infty}$ (ng h/ml)	2660
$t_{1/2}$	15.0
t_{max} (h)	4.0
k_{el}	0.0462

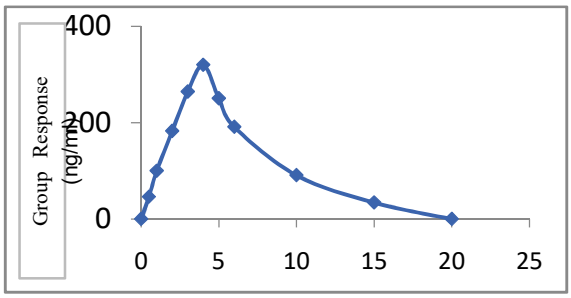

Fig.13: Mean plasma concentration versus time graph of Ripretinib in LCMS/MS

DISCUSSION

A simple, novel Bioanalytical method was developed using LC-ESI-MS/MS and validated for Ripretinib in rat plasmaand found that all the results were within the specifications. As the literature survey revealed that there were no analytical works carried out till date, hence the developed method can be employed for determining concentration of Ripretinib in biological fluids. This work can be further extended to carry out Bioequivalence studies.

CONFLICTS OF INEREST

NIL

ACKNOWLEDGEMENT

We are thankful to the University College of Pharmaceutical Sciences Acharya Nagarjuna University and Shree Icon Pharmaceutical Laboratoriesfor providing facilities and guidance in carrying out the work.

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