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Bioanalytical Method Development and Validation for the Estimation of Ripretinib and its Pharmacokinetic Study in Rat Plasma by LC-MS

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1. Abstract

In this study, sensitive liquid chromatography coupled with the tandem mass spectrometry (LC-MS/ MS) method was developed and validated for the determination of Ripretinib in rat plasma. Liquid-liquid extraction using dichloromethane allowed successful separation of the analyte and internal standard (IS) Sorafenib on an Agilent Zorbax SB C18column with a mobile phase consisted of a mixture of Tetrahydrofuran: 0.2% formic acid solution (80:20, % v/v) delivered at a flow rate of 0.9 mL/min. Mass spectrometric detection was operated in positive multiple reaction monitoring (MRM) mode. The ion transitions monitored were 510.09 → 94.06 for Ripretinib, m/z 465.09 → 185.07 for the IS. The chromatographic run time was 20 min per injection, with 7.68 and 16.57 min retention times for ripretinib and IS, respectively. The calibration curves were linear over the concentration range of 1-80 ng/mL (r2 > 0.9987) for ripretinib. The results of the intra- and inter-day precision and accuracy studies were well within the acceptable limits. This method was successfully applied to a clinical pharmacokinetic study in healthy rats after a single oral administration of 3 mg/kg bw of ripretinib. The maximum plasma concentration (Cmax), the time to the Cmax, and the elimination half-life for ripretinib were 44.59 ± 2.11 ng/mL, 4.52 ± 0.61, and 7.29 ± 1.16 h, respectively. Thus, the method is precise and sensitive enough for its intended purpose. The developed assay method was successfully applied to a pharmacokinetic study in human volunteers.

2. Keywords

Ripretinib, LC-MS/MS, IS, m/z, Pharmacokinetics, GIST

3. Introduction

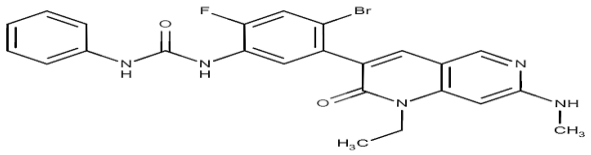
Gastrointestinal stromal tumor (GIST) is a common gastrointestinal cancer, is caused by mutations in KIT and PDGFRA. GIST serves as a model for molecularly Targeted agents, with ripretinib being the latest success. However, resistance often leads to reactivation there oncogenic signaling, highlighting the need for Novel therapeutic mechanism [1].

Ripretinib chemically designated as 3-{4-bromo-5-[1-ethyl-7-(methylamino)-2-oxo-1,2-dihydro-1,6-naphthyridin-3-yl]-2fluorophenyl}-1-phenylurea. Its molecular mass and chemical formula are 510.367g.mol–¹ and $C_{24}H_{21}BrFN_5O_2$, respectively [2]. Ripretinib, sold under the brand name,

Qinlock. It is a medication for the treatment of GIST.

Ripretinib, a switch control kinase inhibitor targets mutant KIT & PDGFRA Kinases in Cancers and myeloproliferative Neoplasms, particularly in Gastrointestinal stromal Tumors [3,4]. It Effectively inhibits all tested mutants and is type-II kinase inhibitor, overcoming the challenge of drug-resistant KIT mutations in GIST.

Figure 1: Structure of ripretinib.



Several analytical methods using LC-MS/MS to quantify various tyrosine kinase inhibitors have been published so far, and most of the assays were developed to determine a single TKI or its metabolites [5]. However, a few methods were able to quantify multiple TKIs simultaneously in various types of biological matrices [6]. Since ripretinib was recently approved, its bio-analysis would be essential for scientific decision-making in new drug development and regulatory filings [7]. Few Analytical methods were reported on ripretinib such as high-performance liquid chromatography with Fluorescence Detection method, UPLD/ MS/MS and HPLC/MS/MS methods [8]. This proposed work aims to develop a method to determine repretinib in rat plasma by LC-MS/MS and pharmacokinetic parameters as well. We developed and validated a simple and sensitive LC-MS/MS method to quantify ripretinib in rat plasma to fulfil this gap [9]. All procedures followed the FDA guidelines for bioanalytical method validation because of the need to perform the preclinical pharmacokinetic study of ripretinib [10].

3. Materials and Methods

3.1. Materials and reagents

The reference sample of Ripretinib (99.59%) was procured from Dr. Reddy's Laboratories, Hyderabad, India, and Sorafenib (99.28%) used as an internal standard (IS) in this study, was obtained from Hetero drugs Ltd, Hyderabad, India. HPLC grade Tetrahydrofuran, acetonitrile, dichloromethane, methanol, and analytical grade ammonium formate were purchased from Merck Specialties Pvt. Ltd., Mumbai, India. All aqueous solutions, including the buffer for the mobile phase, were prepared with Milli-Q (Millipore, Milford, MA, USA) grade water.

3.2. Instrumentation and chromatographic conditions

The study used HPLC system with a Zorbax SB C18 column, LC-20 AD prominence pump, autosampler, and solvent degasser for analytical analysis. Samples were injected into the column and separated using an isocratic mobile phase.

S-MS detection was performed for both analytes and internal standards using an MDS Sciex API-4000 mass spectrometer. Multiple reaction monitoring (MRM) mode was used to identify m/z transition pairs for ripretinib (RTB) and Sorafenib (IS). The analysis data were processed using Analyst software TM (version 1.4.2).

Figure 2a: Product-ion mass spectra of Ripretinib.

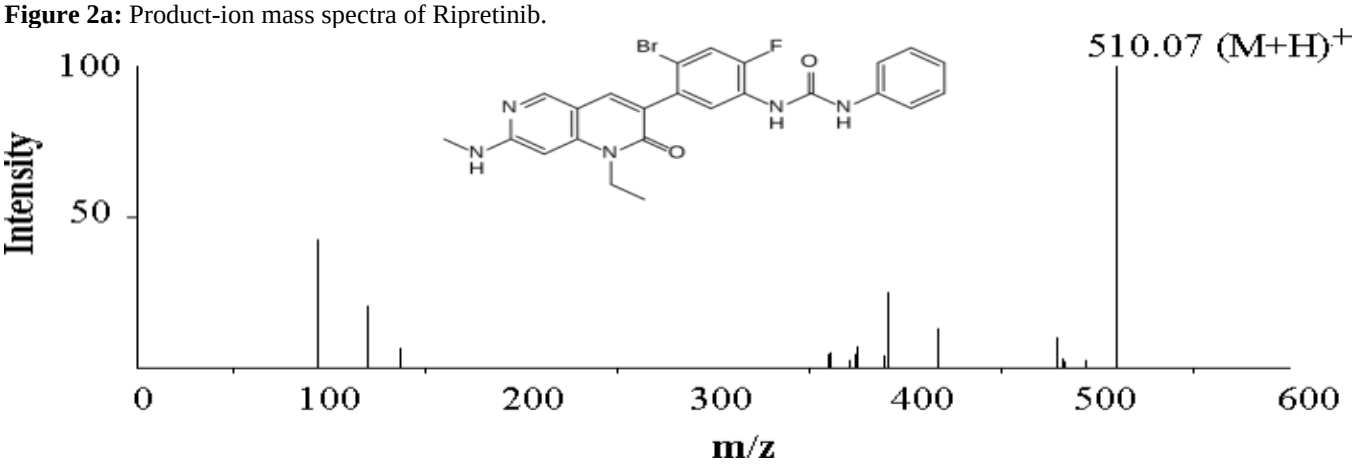
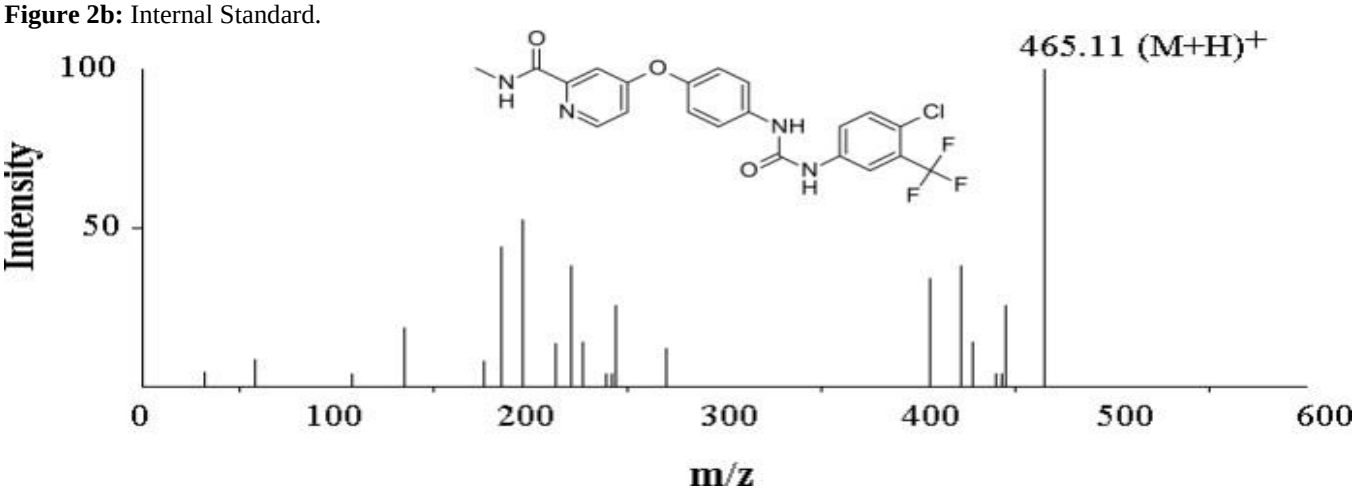


Figure 2b: Internal Standard.



3.3. Preparation of stock and working solutions of analytes and IS

Two stock solutions of RTB and IS were prepared in a mixture of 80:20 THF: water and stored at 2-8 °C. Working standard solutions were prepared by dilution with THF: water. Calibration standards were prepared by spiked RTB to blank rat plasma and quality control working solutions were prepared from RTB stock solutions. All solutions were stored at 4°C before use.

3.4. Processing of calibration and quality control samples

A high-throughput liquid-liquid extraction method was developed for ripretinib sample preparation, with dichloromethane being the best solvent for extraction. The method involves adding IS working standard, formic acid and extracting the analyte and IS from plasma. The samples are vortexed, centrifuged, and evaporated before being reconstituted and transferred to HPLC vials for further analysis using LC-MS/MS.

3.5. Method validation: The method was validated for the fundamental validation parameters following the guidelines of the USFDA and the International Council for Harmonization (ICH) guidelines.

3.5.1 Selectivity: The selectivity or specificity was evaluated by comparing the chromatograms of blank plasma samples collected from six untreated rats with those of spiked plasma samples at LLOQ and plasma samples collected 1-hour post-administration from rats that received intragastric ripretinib. Any interference from unwanted plasma components at the elution time of RTB and IS was evaluated by observing the peak characteristics.

3.5.2 Linearity and lowest limit of quantification (LLOQ): Linearity was assessed for RTB in the concentration range of 1-80 ng/mL (1, 2, 4, 8, 14, 20, 40, and 80 ng/mL) at eight level spiked plasma concentrations. The calibration curve was constructed by plotting the peak area ratios (y) of the analytes to IS against the spiked concentrations of the analytes (x) with a 1/x2 weighted linear least-squares regression and the regression coefficient (r2) was determined. The lowest concentration at which the signaltonoise ratio was ≥ 10 with acceptable accuracy (percent relative error ≤ 20%) and precision (RSD ≤ 20%) was considered the LLOQ. It was determined by the analysis of six replicates of LLOQ samples in three separate validation batches.

3.5.3 - accuracy and precision: The intra-day and inter-day assay accuracy and precision were assessed by analyzing six replicates of different Quality control samples, with a limit of ± 15% for samples outside the LLOQ, and ± 20% at the LLOQ for accuracy and precision.

3.5.4 Extraction recover and Matrix Effect: The study analyzed extraction recoveries of RTB and IS in rat plasma samples at different concentration levels. Samples were extracted and spiked with analyte solution concentrations. The matrix effect of RTB was tested in six different sources of rat plasma at different concentration levels. The recovery of IS was evaluated at a final concentration.

3.5.5 Stability: The analytes stability was evaluated by analyzing four QC samples at low, medium-1, medium-2, and high QCs in six replicates, exposed to different storage and handling conditions, including storage at room temperature for 24 h (bench-top stability), freezer storage at -80 °C for 1 month (long-term storage stability), and storage in the instrument autosampler at 10°C for 16 h (post-preparative/autosampler stability). In addition, freshly prepared processed samples were subjected to three cycles of freezing at -80 °C and thawing at room temperature to characterize the analytes' freeze-thaw stability. These results were compared with the nominal values expressed in stability (%) and RSD (%).

3.6. Pharmacokinetic interaction study

Pharmacokinetic studies were carried out using male Sprague-Dawley (SD) rats (weight 230-250 g) in PGP life sciences, Hyderabad, as per the IAEC approval file no. IAEC/PGP-LS0879/APPROVED/L-03/2020-21. Animals were bred in a breeding room for a week with the room temperature of 21-23°C and humidity of 30-60%, then fasted overnight (12 h) before the experiment. Six SD rats received intragastric administration of a single dose of 3 mg/kg bw of RTB. Blood samples were collected in heparinized tubes before dosing and at 0.5, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 8, 12, 24, 36, and 48 hrs post-dosing. All blood samples were centrifuged immediately at 3500 rpm for 10 min, and the collected plasma samples were stored at -20°C until analysis. All procedures followed the National Institute of Health's guidelines regarding the principles of animal care (2004).

3.7. Statistical Analysis

The main pharmacokinetic parameters of RTB were calculated using non-compartmental analysis with Phoenix WinNonlin 6.2.1 kinetic software. All values were reported as mean ± standard deviation. The PK parameters between the combination group and the single drug group were compared in paired t-test. A value of $P < 0.05$ was considered statistically significant, and $P < 0.01$ was very significant.

4. Results and Discussion

4.1 Method development

4.1.1. Optimization of Sample Preparation: An efficient sample preparation process could achieve the desired recovery and eliminate the interference from endogenous components from the matrix. Since ripretinib was very recently authorized by USFDA, solid phase extraction procedure for the molecules has been discovered to be published. Initial attempts of the SPE methods using various solid-phase containing cartridges such as Oasis Prime HLB, Oasis HLB, and Oasis MCX were failed in successfully extracting RTB and IS from spiked blank plasma samples.

Therefore, the liquid-liquid extraction (LLE) approach was used to establish a new and effective procedure for extracting analyte and IS. Moreover, LLE offers a high capacity of the analyte and high selectivity of separation. Among the different solvents, methyl tertbutyl ether (MTBE), diethyl ether, dichloromethane, and ethyl acetate tested, dichloromethane was the best choice for the separation of RTB. The recovery for the analyte and the IS were excellent and reproducible.

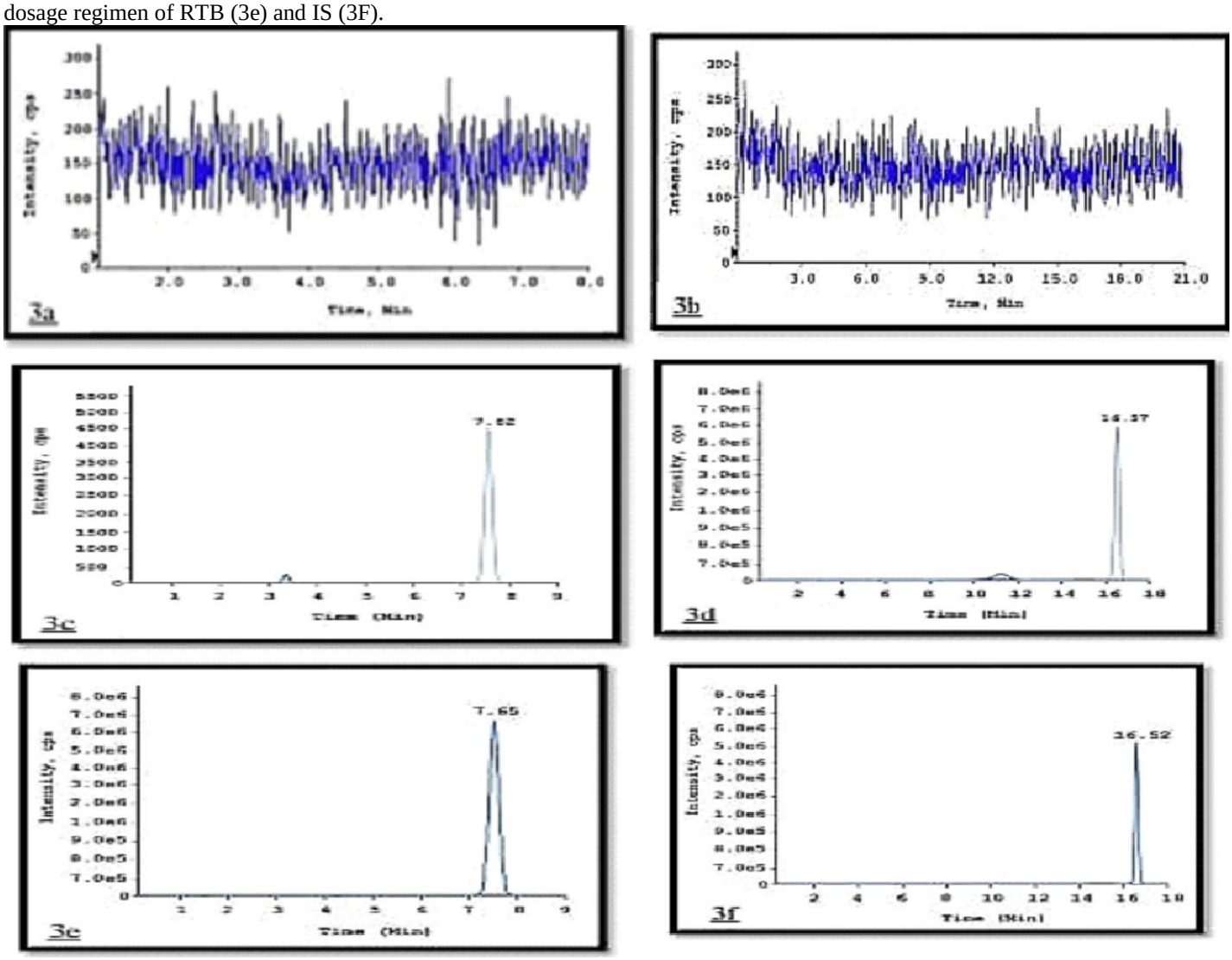
4.1.2. Acceleration of MS/MS Method: The study optimized mass spectrometry and liquid chromatography for the detection of RTB and IS using quadrupole full scans in

both positive and negative ion detection modes. The ion transitions monitored for quantification were m/z 510.09/94.06 for RTB and m/z 465.09/185.07 for IS. The study used a Zorbax SB C18 column and a pre-column Eclipse XDB to separate RTB and IS with high resolution. The best resolution and sensitivity were achieved with an isocratic elution and a mobile phase.

4.2. Method validation

Selectivity: The representative chromatograms of blank plasma samples (**Figure 3a**, and **Figure 3b**) and extracted blank plasma samples at LLOQ (**Figure 3c**, and **Figure 3d**) and extracted plasma samples collected from rats after 1 h after intragastric administration (**Figure 3e**, and **Figure 3f**) of RTB and IS at their respective retention times were shown in **Figure 3**. The RTB and IS peaks were well separated, and no significant interferences from the endogenous plasma components were observed at the analytes and IS retention times. The method was found capable of separating and quantifying RTB in the presence of other plasma components.

Figure 3: Typical MRM chromatograms representing blank plasma analysis at the retention times of RTB (3a), IS (3b); blank plasma extracted samples at LLOQ of RTB (3c); IS (3d); extracted plasma samples collected after 1h of post-administration of dosage regimen of RTB (3e) and IS (3F).



4.2.1. Calibration curve and LLOQ: The plasma calibration curves were linear (r2 > 0.9996) over the concentration range of 1-80 ng/mL for RTB. The calibration curve was prepared by determining the best fit of peak-area ratios (peak-area of analyte/peak-area of IS) versus analyte concentration and fitted to the y = mx + c (**Figure 4**). The regression equations of RTB were calculated as y = 0.0234x - 0.015. The LLOQ of RTB was 1 ng/mL, with a precision of 4.01 (% RSD) and accuracy of -3.33 (% RE) (**Table 1**). **Figure 3c** depicts representative chromatogram of 1 ng/mL RTB.

4.2.2. Precision and Accuracy: 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 within 15% of the relative standard deviation (RSD) at low, middle-1, middle-2, and high-quality control levels, whereas within 20% at LLOQ QCs. The intra-day and inter-day accuracy (RE%) and precision (%RSD) of RTB were in the range of -3.55 to 0.63 and 3.54 to 9.08, respectively, at overall levels of quality control samples, including LLOQ. Thus, the results revealed good precision and accuracy of the developed LC-MS/MS method.

4.2.3. Recovery and matrix Effect: The extraction recovery and matrix effect results are shown in **Table 2**. The extraction recoveries for RTB at LQC, MQC-1, MQC-2, and HQC levels were 98.11 ± 2.87, 94.38 ± 2.31, 91.28 ± 1.89, 92.55 ± 2.48 ng/mL respectively. The mean extraction

recovery of IS was 89.47 ± 3.15 % (n = 12). The results showed that the developed method had good extraction efficiency, and the recovery of RTB in rat plasma was consistent, precise, and reproducible .IS-normalized matrix effects of RTB were 0.98, 0.99, 0.97, 1.01 0.99 and the % RSDs were 2.45, 3.57, 2.78, 3.86 and 2.69, at LLOQ, LQC, MQC-1, MQC-2 and HQC levels respectively. The above results are all within the acceptance limit, and it was illustrated that the rat plasma matrix had no interference in the analysis of RTB.

4.2.4. Stability: QC samples of RTB at four concentrations LQC, MQC-1, MQC-2, and HQC, were analyzed in six replicates for studying the possible conditions to which the samples might be exposed during storage and handling. It was found that RTB was stable in rat plasma after being stored at room temperature for 24 h, after repeated three freeze-thaw cycles, and after being stored at -70 °C for 30 days. In addition, the treated samples were found to be stable in the autosampler for a period of 24 h, and the results were found to be within the assay variability limits during the entire process. All results of the stability tests are summarized in **Table 3**.

Figure 4: The linearity graph of Ripretinib in spiked rat plasma over the concentration range of 1 to 80 ng/ml.

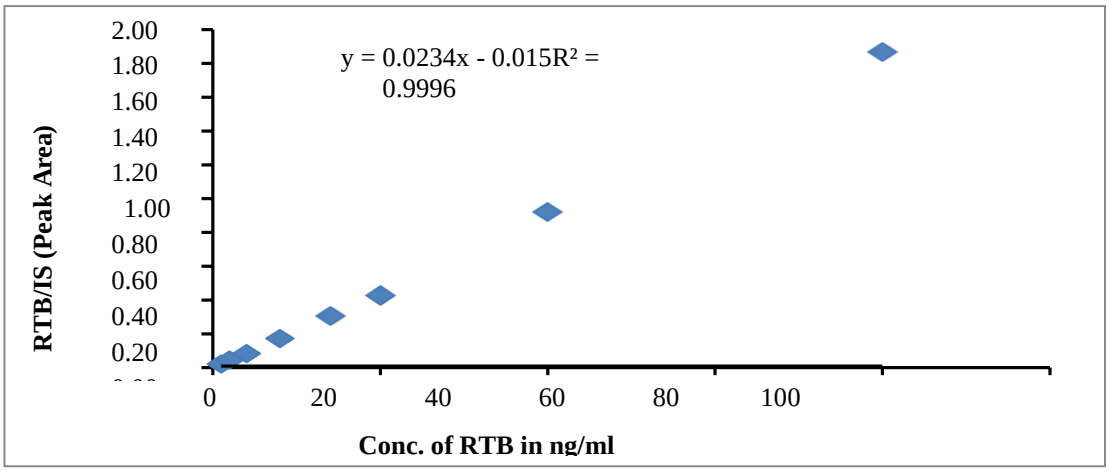


Table 1: LLOQ, Accuracy, and precision of RTB in spiked rat plasma by LC-MS/MS method.

Analyte	QC level	Nominal concentration In ng/mL	Intra-day (n=6)			Inter-day (n=18)		
			concentration (ng/mL)	RSD (%)	RE (%)	concentration(ng/mL)	RSD (%)	RE (%)
Ripretinib	LLOQ	1	0.93± 0.16	4.01	-3.33	0.89± 0.2	3.60	-0.97
	LQC	3	2.91 ± 0.36	3.54	0.63	3.08 ± 0.47	5.28	-0.13
	MCQ1	12	11.89 ± 1.74	5.11	-0.34	11.93 ± 1.58	9.08	-0.10
	MCQ2	35	34.92 ± 1.92	8.04	-0.04	34.87 ± 1.64	7.02	-0.02
	HQC	65	64.86 ± 1.85	6.23	-3.51	64.91± 1.96	4.36	0.54

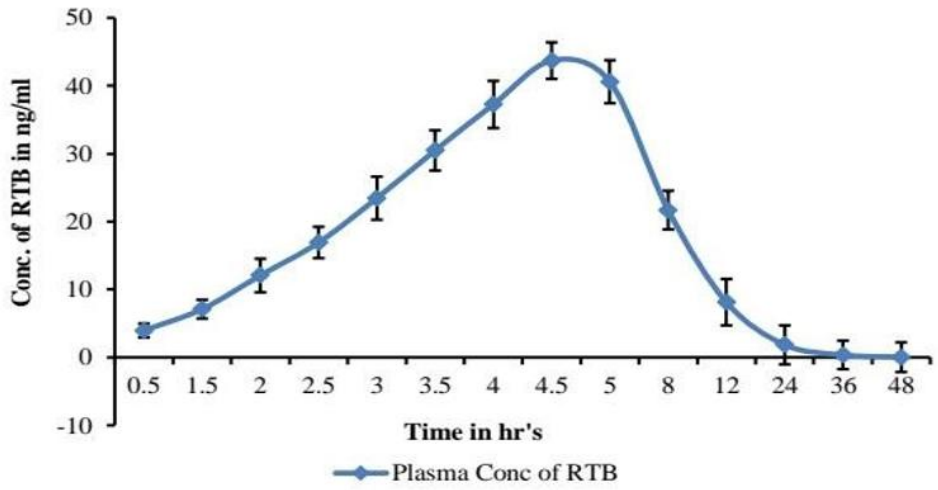
Table 2: Results of extraction recovery and matrix effect of ripretinib.

Analyte	Measurement Level	NominalConc. In ng/mL	Extraction Recovery		Matrix effect	
			Recoveries in % (Mean ± SD)	RSD(%)	IS-Normalized matrix factor	RSD(%)
RTB	LLOQ	1	93.47 ± 2.15	2.56	0.98	2.45
	LQC	3	98.11 ± 2.87	1.88	0.99	3.57
	MQC1	12	94.38 ± 2.31	3.72	0.97	2.78
	MQC2	35	91.28 ± 1.89	1.76	1.01	3.86
	HQC	65	92.55 ± 2.48	1.78	0.99	2.69
IS	-	100	89.47 ± 3.15	2.93	-	-

Table 3: Stability results of ripretinib.

Analyte	Measur ement Level	Nomin al Conc. In ng/mL	Room temp. For 24hrs		-80C for 1month		Autosampler for 16 hrs		Freeze-Thaw Cycles	
			Stability (%)	RSD (%)	Stability (%)	RSD (%)	Stability (%)	RSD (%)	Stability (%)	RSD (%)
RTB	LLOQ	1	103.4	2.56	105.1	4.16	97.41	3.29	106.5	4.59
	LQC	3	94.27	3.28	95.2	3.79	106.29	4.06	94.4	4.12
	MQC1	12	97.88	2.89	91.47	5.03	111.32	3.85	91.3	3.68
	MQC2	35	106.3	3.78	106.7	4.37	92.5	2.47	109.5	2.75
	HQC	65	101.6	2.94	99.5	3.74	105.6	4.26	97.1	3.55

Figure 5: Mean plasma concentration-time profile of RTB in rat plasma following oral administration of 3 mg/Kg bw.



4.3 Pharmacokinetic study

In order to verify the sensitivity and selectivity of this method in a real-time situation, the present method was used to examine the pharmacokinetic status of RTB in Sprague Dawleyrats (n = 6). The primary pharmacokinetic parameters viz., maximum plasma concentration (Cmax) and area under the plasma concentrations versus time curve (AUC) ranging from time zero to last measurable concentration (AUC0-t) and extrapolated to infinity (AUC0-∞) were compared by an analysis of variance (ANOVA). These pharmacokinetic parameters, including Tmax (h), t1/2 (h) of RTB, are illustrated in **Table 4**. The maximum plasma concentration (Cmax) of RTB escalated to 44.59 ± 2.11 ng/mL at Tmax of 4.52 ± 0.61 h, which was kinetically correlated with the reported data (13). The mean areas under the concentration-time curve (AUC0-48 h) and concentration-infinity time curve for RTB were 5917.17 ± 1365.31 ng h/mL and 6156.25 ± 1654.23 ng h/mL, respectively. The elimination half-life (t1/2) of RTB was determined using the kinetic study and was 7.29 ± 1.16 h, which agreed with the published study. The present study revealed that the intragastric administration of RTB shows clinical acceptability (**Figure 5**).

Table 4: Key pharmacokinetic parameters of ripretinib.

Parameter	Ripretinib
Cmax (ng/mL)	44.59 ± 2.11
tmax (h)	4.52 ± 0.61
t1/2 (h)	7.29 ± 1.16
AUC0-t (ng h/mL)	5917.17 ± 1365.31
AUC0-∞ (ng h/mL)	6156.25 ± 1654.23

5. Conclusion

A sensitive liquid-liquid extraction-based LC-MS/MS method was developed and validated to determine Ripretinib in rat plasma successfully. We have developed and validated an LCMS/MS method that can successfully quantitate Ripretinib in rat plasma. The method was fast, selective and sensitive; the drug extraction process was simple; the run time was short; the LLOQ of RTB was 1 ng/mL. To our knowledge, this is the first report on the pharmacokinetics in rats after an oral administration of 3 mg/ kg.bw ripretinib under fasting conditions. The pharmacokinetic parameters were collected for further investigation. This method can be used for therapeutic monitoring of ripretinib and evaluating the possible impact on the therapeutic Efficacy for treating gastrointestinal stromal tumors.

6. Conflict of Interest

The authors have no conflicts of interest regarding this investigation**.**

7. Acknowledgments

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