RESEARCH ARTICLE

Bio-analytical Methodology for Exploring Ripretinib's Pharmacokinetic Profile in Rat Plasma Employing Bosutinib as an Internal Standard Utilizing LC-MS/MS

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Received: 05th April, 2024; Revised: 01st May, 2024; Accepted: 06th May, 2024; Available Online: 25th June, 2024

ABSTRACT

A robust, consistent, straightforward, swift, and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was tailored for the bioanalytical analysis of ripretinib, with bosutinib serving as the comparative standard. This article provides a synopsis of recent developments in bioanalytical LC-MS/MS techniques utilizing a phenyl column (150x4.6 mm, 3.5 µ) and an eluting agent composed of ammonium formate at a pH of 2.5, which was altered in a 50:50 ratio with formic acid and acetonitrile. The chromatogram was utilized to analyse ripretinib, and the MS detector was equipped with electrospray ionization (ESI) method functioning in positive ionization conditions with multiple reactions monitoring (MRM). This allowed for the recording of transition m/z values of 531.4465→170.9638 for ripretinib and 511.4230 → 160.7133 for BTB. In the chromatographic analysis, ripretinib was retained for approximately 2.433 minutes, whereas BTB was retained for around 3.608 minutes. The validity of the approach extends across a linearity scale of 5 to 100 ng/mL for ripretinib, boasting a correlation index of 0.9999. In the realm of biological matrices, the extraction procedure employing the protein precipitation technique yields recovery rates of 99.6, 99.2, and 98% at the lower, middle, and higher quality control levels, respectively. The obtained results for accuracy, precision, matrix effect, and stability were determined to be within the acceptable range. To observe the investigated analyte in bodily fluids, pharmacokinetic studies yielded key parameters, including a half-life of 10 hours and a time to reach a maximum drug level of 4 hours. This thorough assessment affirms that the method satisfies rigorous criteria for system suitability and specificity well within predefined acceptance limits. Such performance positions it as an indispensable asset in the realm of bioanalysis, significantly broadening its clinical applicability.

Keywords: Ripretinib, LC-MS/MS, Bioanalytical validation, Rat plasma, Pharmacokinetic studies.

International Journal of Pharmaceutical Quality Assurance (2024); DOI: 10.25258/ijpqa.15.2.11

How to cite this article: Pilli S, Kalakonda SN, Rajendran V. Bio-analytical Methodology for Exploring Ripretinib's Pharmacokinetic Profile in Rat Plasma Employing Bosutinib as an Internal Standard Utilizing LC-MS/MS. International Journal of Pharmaceutical Quality Assurance. 2024;15(2):626-630.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Ripretinib (RTB) chemically known as 1-[4-bromo-5-[1-ethyl-7-(methylamino)-2-oxo-1,6-naphthyridin-3-yl]-2-fluorophenyl]-3-phenylurea, is marketed under the brand name Qinlock. Refer to Figure 1 for its structural representation. RTB (DCC-2618) offers a promising solution for patients with gastrointestinal stromal tumors (GIST) who have experienced progression on authorized medications as a result of the emergence of additional KIT resistance mutations.[1] These mutations are highly heterogeneous, and existing tyrosine kinase inhibitors (TKIs) may not fully cover them. Consequently, serial application of TKIs, such as RTB has become a common strategy in advanced GIST treatment. RTB exhibits a unique dual mechanism of action.[2] Unlike other agents targeting GIST, which bind to the adenosine triphosphate (ATP)-binding site of the kinase, RTB targets both the kinase switch pocket and the activation loop. By doing so, it effectively turns off the kinase, thereby inhibiting dysregulated cell growth. This broad mechanism allows RTB to restrain the multiplication of both common strain and variant forms of GIST, including genetic alterations in KIT Coding segments 9, 11, 13, 14, 17, and 18 and PDGFRA coding segments 12, 14, and 18. Moreover, RTB's inhibitory effects extend beyond KIT and PDGFRa.[3] It also blocks several other kinases involved in tumor cell growth, including vascular endothelial growth factor receptor type 2 (VEGFR2), angiopoietin-1 receptor (TIE2), PDGFR-beta, and macrophage colony-stimulating factor 1 receptor (CSF1R).[4] This broader spectrum of inhibition may contribute

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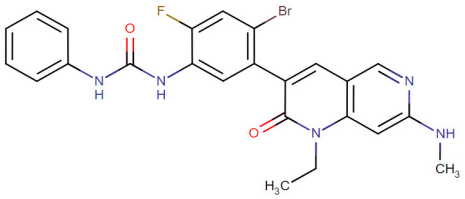

Figure 1: Molecular configuration of RTB

to its efficacy in inhibiting tumor growth and angiogenesis in GIST sufferers. The most regular complications include hair loss, exhaustion, queasiness, abdominal discomfort, bowel obstruction, muscular discomfort, loose stools, reduced hunger, palmar-plantar erythrodysesthesia syndrome, and vomiting.[5] Clinical studies have shown a notable enhancement in progression-free survival (PFS) through RTB therapy, with both statistically substantial and practical significance being observed. This indicates that RTB effectively delays disease progression compared to other therapies.[6] RTB is associated with a favorable side effect profile, which is an important consideration in its selection as standard therapy.[7] This suggests that RTB is generally well-tolerated by patients, leading to improved treatment adherence and quality of life. Despite these strengths, it is unclear what the ideal line of therapy for RTB should be in the treatment sequence for advanced GIST patients.[8] Identifying the resistance mechanisms that develop with RTB therapy and developing effective strategies for managing progression on RTB are critical for optimizing patient outcomes. Analyzing RTB is essential for comprehending its overall impact on patient's well-being beyond just clinical endpoints, thereby elucidating the efficacy of RTB therapy. At present, the literature contains records of solely one HPLC approach[9] and one UPLC-MS/MS method[10] designated for the analysis of RTB. Therefore, the author has endeavored to evolve a bioanalytical strategy for analyzing pharmacokinetic aspects of RTB. This endeavor is crucial for advancing scientific understanding, optimizing treatment strategies, and ultimately enhancing outcomes for patients with advanced GIST. We are confident that this marks the initial strides in developing a bioanalytical LCMS approach specifically tailored for analyzing RTB, utilizing BTB as an internal standard.

MATERIALS AND METHODS

Substances and Reactants

Acetonitrile, ammonium formate, formic acid, and ultra-pure water was acquired from Merck Pharmaceuticals Ltd, positioned in Worli, Mumbai, India. RTB and BTB, serving as reference standards, were obtained from Zydus Cadila Healthcare Ltd, based in Secunderabad.

LC-Ms Equipment and Settings

The bio-analytical assay was developed using the liquid chromatography framework (Waters Alliance pattern) incorporating a QTRAP 5500 triple quadrupole mass spectrometer instrument (SCIEX), featuring an electrospray ionization interface. The carrier phase flow velocity of 1-mL/min exhibits high responsiveness in the positive ionization approach, ensuring detectability and signal constancy. Ion pair tracking was conducted using MRM mode: m/z 511.4230 → 160.7133 for RTB and m/z 531.4465→170.9638 for BTB as depicted in Figure 2. Operating parameters included an ion generation potential of 5500V, spray chamber temperature of 550°C, gas evaporation temperature ranging from 120 to 250°C, fragmentation gas nitrogen at a pressure of 55 psi, desiccation gas flow speed of 5 mL/min, ion clearance potential of 40V, inlet voltage of 45V, departure potential of 15V, ionization voltage of 5500V, and a residence period of 1-second.

Chromatographic Settings

Chromatographic separation was carried out at room temperature under isocratic conditions using a phenyl column (150 x 4.6 mm x 3.5 µm). As a carrier phase, a mix of ammonium formate of pH-2.5 adjusted with formic acid and acetonitrile at 50:50 v/v with a flow of 1.0 mL/min was used. The infusion of 10 µL of the sample was followed by a 5 minutes cycle. Throughout the analysis, the column temperature remained consistent with the standard setting. BSB was selected as the internal standard because of its favorable chromatographic performance and extractability characteristics.

Establishment of calibration and quality control standards

The analytical standards of RTB and BSB were formulated at the strength of 1-mg/mL. Following this, the RTB standard solutions, diluted suitably from the master stock solution, were introduced into drug-free rat plasma. This yielded RTB concentrations of 5, 12.5, 25.00, 37.5, 50, 62.5, 75, and 100 ng/mL, forming the analytical calibration standards. Moreover, QC standards were set up with specific RTB concentrations of 5, 25, 50, and 75 ng/mL. These quality control samples serve the purpose of monitoring the development

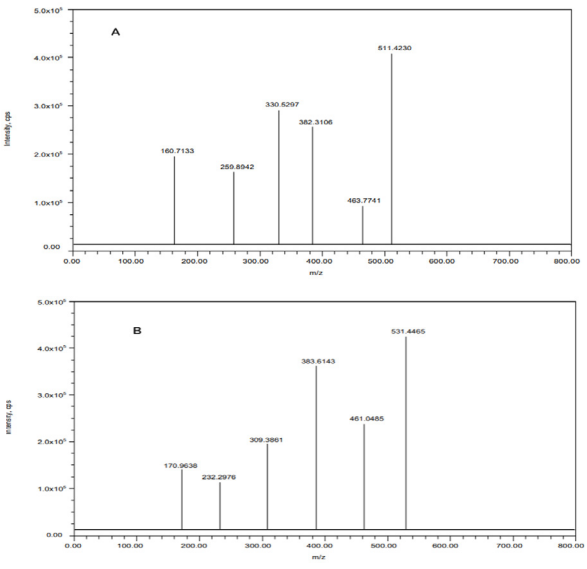

Figure 2: Mass spectral profiles of (A) RTB and (B) BTB

and reliability of the analytical method. All calibrated and conformance standards were carefully cold-stored and maintained at -30°C for preservation.

Extraction of RTB and BSB from biological matrix

The protein precipitation method was utilized to extract RTB and its corresponding internal standard BSB from rat plasma samples. Initially, 500 µL of the internal standard (IS) stock solution was combined with 200 µL of plasma at equivalent concentrations, ensuring thorough mixing. Next, 300 µL of acetonitrile was included, and the blend was vigorously vortexed for 10 minutes. Subsequently, the mixture underwent centrifugation at 5000 rpm for 20 minutes, resulting in the separation of the upper layer. The solution was then gathered and clarified through a 0.45 µ nylon syringe filter. The resulting pure solution was relocated into an ampule and delivered into a chromatographic apparatus.

Bio-analytical Method Validation

Selectivity

Retention times of RTB and the BSB were determined, and potential contamination from unanalyzed samples was evaluated by analyzing rat blood plasma specimens from six different rats to assess selectivity. A set criterion was established to verify selectivity. The peak area of any potential interfering compounds at their specified retention times in matrix blanks should not exceed 20% of the average peak response of the compound's LoQ. Additionally, the average peak response of the IS should not exceed 5% of the peak area at its retention time in blank samples.

Matrix effect

To evaluate the effect of matrix on RTB, we compared analyte elution fraction in isolated plasma fractions from six independent plasma fractions devoid of drug and slick recovery samples. Six different lots of plasma were tested at both LQC and HQC levels in triplicate. To satisfy the acceptance criteria, a minimum of 67% (2 out of 3) of samples at each level must be within the range of 85.00 to 115.00%. Moreover, a minimum of 80% (5 out of 6) of the matrix lots must satisfy the acceptance criteria.

Recovery

The method of determining recovery involved a comparison of peak areas from standards that hadn't undergone extraction to those of RTB that had been extracted (with six replicates per QC concentration). The QC samples encompassed LQC, MQC, and HQC, each having concentrations of 25, 50, and 75 ng/mL, respectively.

Linearity

This research demonstrates the linearity of detector response through the utilization of eight distinct concentrations of an RTB solution, spanning from 5 to 100 ng/mL. The selected concentration range was deliberate and aimed at showcasing the detector's linear response. The experimental protocol employed an extraction method consistent with validating the detector's linearity while evaluating diverse concentrations of RTB solutions introduced into rat plasma. A fixed concentration of 50 ng/mL was maintained for the internal standard. Subsequently, the HPLC system was supplied with the determined sample concentrations. The acquired data, representing the ratio of RTB peak magnitude to BSB peak magnitude, was utilized to construct a correlation plot correlating the ratio with PC concentration in ng/mL. Regression analysis was then conducted to ascertain crucial parameters, including the correlation index, incline, intersection point, LoD, and LoQ.

Precision and accuracy

Quality validation duplication examination was conducted on six samples to evaluate outcomes across three distinct quality control levels: low, medium, and high. Using the area response ratio values that resulted from comparing measured responses to expected real responses at each QC level, the RSD was computed. It's important to remember that the CV at all other QC levels should be less than 15%, except the LLoQ, where it should be below 20%.

Stability

The stability of the stock solution was determined by comparing the region response of the fresh stock solution sample to the stability samples' analyte area response. Six dosage repeats assessed plasma stability using LQC and HQC concentrations. The USFDA defines stability as an analyte CV below 15%. A 24-hour bench-top stability test was performed on injected rat plasma samples maintained at room temperature. Plasma samples were either injected immediately or stored in the autosampler for a day at 2 to 8°C to verify its stability. Freeze-thaw stability was tested by comparing newly infused quality control samples to those frozen at -30°C and thawed three times. The 24-hour concentration was compared to the starting concentration to determine long-term stability.

Pharmacokinetic study

The procedural protocols concerning animal experimentation were formally presented to the Committee for Control and Supervision of Experimentation on Animals (CPCSEA). Following the presentation, approval was secured from the Institutional Animal Ethics Committee ((Reg.No:1074/PO/Re/S/23/CPCSEA). Test subjects consisted of 6 male Sprague-Dawley rats sourced from Hyderabad-based Biological E. Prior to experimentation. All animals underwent an overnight fast with free access to water. After oral dosing of RTB, blood specimens (300 µL) were drawn from the fossa orbitalis vein at predefined intervals: 0.5, 1, 2, 4, 6, 8, 10, 15, and 20 hours. These samples were gathered in eppendorf tubes incorporating a 10% EDTA solution and whisked at 5000 rpm for 30 minutes at a heat level range of 2 to 8°C. The resulting clarified supernatant plasma was then maintained at -30°C until examination. After concluding the study, the animals were brought back to the animal facility for rehabilitation. The pharmacokinetic variables of RTB were computed using WinNonlin (Version 5.2) software.

Table 1: Precision and accuracy of RTB

QC Level	LLQC (5 ng/mL)	LQC (25 ng/mL)	MQC (50 ng/mL)	HQC (75 ng/mL)
Mean ± SD	0.231 ± 0.005	1.175 ± 0.009	2.413 ± 0.008	3.616 ± 0.007
%CV	2.03	0.74	0.35	0.22
Accuracy	94.48	96.11	98.69	98.60

RESULTS AND DISCUSSION

Precision and Accuracy

The accuracy and precision were evaluated by consolidating all individual assay outcomes from various internal reference samples. The data indicated that the strategy employed was both definite and efficient. Precision results for RTB are presented in Table 1.

Linearity

The chromatographic response ratio of calibration standards demonstrated a commensurate correlation with their concentrations, covering from 5 to 100 ng/mL. The verification graphs displayed a linear relationship, with a correlation coefficient of 0.999 observed for RTB.

Recovery

The evaluation of RTB recoveries at LQC, MQC, and HQC levels demonstrated excellent extraction efficiency in the bioanalytical method. Additionally, the findings indicated consistent recovery across various concentrations, as outlined in Table 2.

Matrix Impact

The %RSD for internal variation in the signal showed 1.0 percent concerning RTB in LC-MS/MS. This indicates that within this scenario, the influence of the matrix on the ionization of analytes falls within an acceptable spectrum of ionization, where ion suppression or enhancement was noted. During the matrix effect assessment, the %mean accuracy at LQC and HQC levels for RTB were recorded as 97.5 and 98.6, respectively with the %CV of 1.18 and 0.29. This suggests that the matrix's influence on the ionization of the analyte remains within acceptable bounds.

Table 2: RTB recovery study data

	Extracted response	*Un-extracted response*	*Matrix factor*
HQC (75 ng/mL)			
Mean ± SD	3.603 ± 0.01	3.615 ± 0.008	0.99660.002
%CV	0.28	0.22	0.16
%Mean recovery		99.66	-
MQC (50 ng/mL)			
Mean ± SD	2.393 ± 0.009	2.411 ± 0.008	0.9927 ± 0.002
%CV	0.37	0.33	0.22
%Mean recovery		99.2	-
LQC (25 ng/mL)			
Mean	1.152 ± 0.008	1.176 ± 0.009	0.9799 ± 0.008
%CV	0.69	0.79	0.83
%Mean recovery		98	

Selectivity

Chromatographic graphs of both control and reference samples are depicted in Figure 3, and notably, no contaminant spikes were exhibited, demonstrating the absence of unwanted compounds.

Stability

RTB solutions were formulated with dilution media for solution integrity assessment and maintained in a cold environment at 2 to 8°C. Newly prepared stock solutions were linked with stock solutions that had been prepared 24 hours prior. The plasma remained stable on the bench-top for 24 hours at room temperature and for 24 hours at 20°C in the autosampler. It was evident from subsequent stability testing that RTB remained stable when maintained at a temperature of -30°C for up to 24 hours. The comprehensive stability findings for RTB are presented in Table 3.

In-vivo Pharmacokinetic Evaluation

The plasma concentration-time profiles of RTB in rats, reveal a characteristic bell-shaped curve for experimental formulations. Notably, RTB remained detectable in the bloodstream for up to 15 hours following oral administration, underscoring the formulation's efficacy in releasing the drug. The pharmacokinetic parameters were meticulously calculated, with the corresponding data summarized in Table 4.

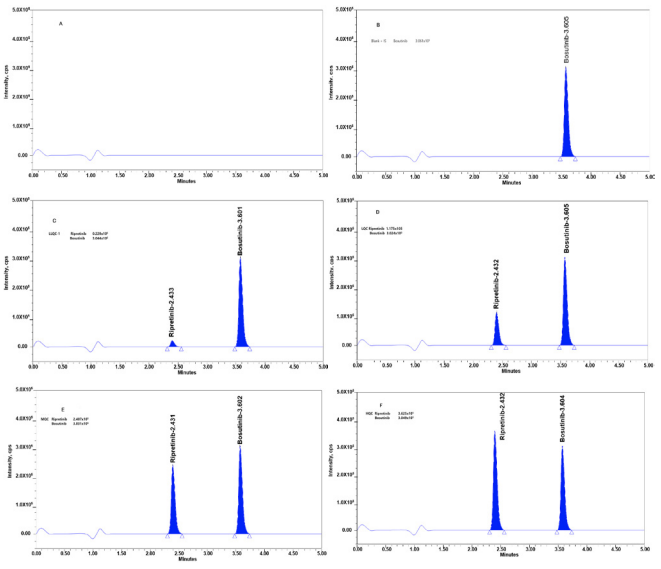

Figure 3: A) Clean plasma chromatogram without interferences; B) Plasma containing internal standard. Exemplary chromatograms of rat plasma samples spiked with RTB; C) (LLoQ): 5 ng/mL; D) (LQC): 25 ng/mL; E) (MQC): 50 ng/mL; and F) (HQC): 75 ng/mL

Table 3: Stability results of RTB

QC level		*Fortified plasma concentration (ng/mL)*	*%Accuracy*	*%CV*
Workbench stability	LQC	25	95.13	0.80
	MQC	50	97.71	0.40
	HQC	75	98.43	0.17
Injection system stability	LQC	25	95.13	0.66
	MQC	50	97.75	0.49
	HQC	75	98.49	0.19
Long term (Day28) stability	LQC	25	83.76	0.84
	MQC	50	84.50	0.51
	HQC	75	86.35	0.28
Durability of wet extracts	LQC	25	94.23	0.56
	MQC	50	97.10	0.24
	HQC	75	97.75	0.25
Consistency of dried extracts	LQC	25	94.23	0.58
	MQC	50	96.32	0.25
	HQC	75	97.83	0.31
Freeze-thaw tolerance	LQC	25	94.89	0.60
	MQC	50	97.71	0.15
	HQC	75	98.43	0.15
Brief-duration steadiness	LQC	25	93.66	0.75
	MQC	50	94.48	0.23
	HQC	75	95.81	0.25

Table 4: Pharmacokinetic parameters of RTB

Parameters	*Mean ± SD*
C_{max}	45.8 ± 1.16 ng ml^{-1}
T_{max}	4.0 ± 0.003 h
C0	20.1 ± 0.6
K_e	0.07 ± 0.002 $^{h-}$1
V_d	41.35 ± 0.88 l kg^{-1}
$T_{1/2}$	10 ± 0.23 h
Cl	2.9 ± 0.1 l h^{-1}
AUC_{0-t}	438.4 ± 13.7 ng h m^{l-}1
$AUC_{0-\infty}$	574 ± 27.6 ng h ml^{-1}

CONCLUSION

This paper introduces a method tailored to enhance the pharmacokinetic investigation of RTB in rats, an essential component of RTB's preclinical drug development. Such scrutiny offers valuable insights into RTB's potential role in managing KIT-altered malignant melanoma, promising improved treatment outcomes for affected individuals. The method employs a straightforward extraction process coupled with protein precipitation, yielding consistent and reproducible analyte recovery with minimal matrix effects, showcasing exceptional selectivity. Validated across a strength of 5 to 100 ng/mL in rat plasma, this method boasts a swift chromatography runtime of 5 minutes, facilitating multiple sample analyses within a single day. Precision values ranging from 0.22 to 2.03 and accuracy spanning 95.1 to 98.8% attest to the reliability of this approach. Extensive evaluations have been conducted to ascertain analyte stability in various sample conditions, both neat and in plasma. Remarkably, this bioanalytical strategy, employing LC-MS/MS, marks the pioneering evaluation of RTB's pharmacokinetics.

ACKNOWLEDGMENT

The authors extend their sincere gratitude to the administration of Shri Vishnu College of Pharmacy, Bhimavaram, India, for generously providing access to the facilities that significantly bolstered their research endeavors.

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