RESEARCH ARTICLE

Development of a Bioanalytical Assay for the Selective RET Inhibitor Pralsetinib in Plasma Samples Utilizing Liquid Chromatography-Tandem Mass Spectrometry

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Received: 07th January, 2024; Revised: 21st March, 2024; Accepted: 16th May, 2024; Available Online: 25th June, 2024

ABSTRACT

Pralsetinib is a potent and specific tyrosine kinase inhibitor targeting the RET receptor, used in treating various cancers. This study developed and validated a bioanalytical assay for pralsetinib in plasma using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The internal standard was erlotinib, and protein precipitation with acetonitrile was used for sample preparation. The analytes were separated on an Ultimate® XB-C18 column using gradient elution with ammonium acetate in water and methanol. Both are acidified with 0.1% formic acid. Detection was performed using positive electrospray ionization in the chosen reaction monitoring mode. The assay was validated over a linear range of 2 to 2000 ng/mL, achieving accuracies greater than 90% and precision values below 2%. The method was stable under all tested conditions without significant matrix effects or extraction losses.

Keywords: LC-MS/MS, Validation, Bio-analysis, Pralsetinib, Erlotinib, Internal standard.

International Journal of Drug Delivery Technology (2024); DOI: 10.25258/ijddt.14.2.36

How to cite this article: Pendyala V, Addanki SP, Suryadevara V. Development of a Bioanalytical Assay for the Selective RET Inhibitor Pralsetinib in Plasma Samples Utilizing Liquid Chromatography-Tandem Mass Spectrometry. International Journal of Drug Delivery Technology. 2024;14(2):847-852.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

Cancer, the leading cause of death worldwide, is characterized by uncontrolled cell growth and metastasis. Genomic instability leads to this disease by disrupting genes that govern critical cellular processes. These disruptions alter cellular function, potentially enabling cells to acquire cancerous characteristics.[1-3] As an example of a genetic change that is now addressed with several types of targeted small molecules, consider RET. RET has been found in various cancer forms, including non-small-cell lung cancer (NSCLC), thyroid carcinoma (both papillary and medullary), colon cancer, and other solid tumors.[4-6] Tumor progression in non-small cell lung cancer is significantly influenced by RET activation. Specific mutations can trigger the tyrosine kinase signal transduction pathway, leading to continuous autophosphorylation. Targeting these tyrosine kinases with inhibitors is a therapeutic approach.[7-12] Pralsetinib, an oral RET tyrosine kinase inhibitor, is under phase 1/2 trials for advanced solid tumors with RET fusion.[13,14] Erlotinib, a structural analog of pralsetinib, serves as an internal standard in this study. Chemical structures of Pralestinib and erlotinib were depicted in Figure 1.

The literature review indicated that no methods for estimating pralsetinib in conjunction with other analytes have been described so far. To yet, pralsetinib has not been detected by any reliable bioanalytical method. To estimate both medications in human plasma at the same time, a novel, fast, accurate, and cost-effective liquid chromatography-tandem mass spectrometry (LC-MS/MS) bioanalytical approach is required. Since there is an increasing need for these treatments on a global scale, this method could be utilized to investigate the bioavailability of various dose formulations.

MATERIALS AND METHODS

Solvents and chemicals Dr. Reddy's Laboratories in Hyderabad, India, supplied the pralsetinib and erlotinib

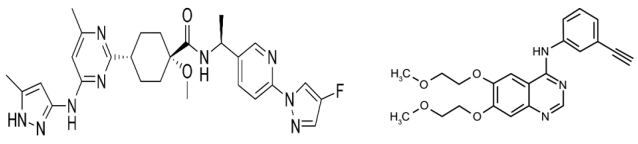

Figure 1: (A) Pralsetinib (B) Erlotinib

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active pharmaceutical ingredients in API form. HPLC-grade solvents and chemicals were sourced from Merck and SD Fine Chemicals, respectively. Human K2-EDTA plasma was procured from a Hyderabad-based Doctor's pathology lab.

Chromatographic Conditions

The solvent phase comprised 5 mM ammonium acetate in 0.1% formic acid and acetonitrile (10:90, v/v), is used in conjunction with an analytical column, the Ultimate® XB-C18 (2.1 × 50 mm, 3.5 µm) with a flowrate of 0.6 mL/minutes. Operating at a flow rate of 0.6 mL/min. The column was maintained at a temperature of 40°C. A 20 µL sample was injected into the LC-MS/MS system. Each injection took three minutes, with the analyte and internal standard eluting at 0.94 and 0.93 minutes for pralsetinib and erlotinib, respectively.

LC-MS/MS Instrument and Conditions

The HPLC system with a triple quadrupole mass spectrometer API 4000 model, produced by Agilent Technologies in Waldbronn, Germany. Data processing was conducted using the analytical 1.4.1 software package (SCIEX). The ion source parameters included gas channels with nebulizer gas at 20 psi, heater gas at 40 psi, curtain gas at 20 psi, and collisional activated dissociation (CAD) at 4 psi. The source temperature was maintained at 400°C with a voltage of 5500 V. Quantitative measurements for pralsetinib and erlotinib were performed using the transitions m/z 534.1/190.4 and 394.2 > 278.1, respectively.

Stock &Working Standard Solutions

To prepare the stock, approximately 25 mg of pralsetinib and erlotinib were dissolved in separate 25 mL volumetric flasks using acetonitrile. The concentrations were then adjusted to achieve a final concentration of 1000 µg/mL for both solutions. Calibration standards with final concentrations of 2, 4, 8, 20, 40, 80, 100, 500, 1000, or 2000 ng/mL were created from these stock solutions. Quality control (QC) samples were prepared at concentrations of HQC: 1800 ng/mL, MQC: 1100 ng/mL, LQC: 6.00 ng/mL, and LLoQQC: 2 ng/mL. Additionally, a working standard solution of the internal standard (IS) at 11000 ng/mL was prepared from a 1-mg/mL IS stock solution. All solutions were stored at temperatures between 2 and 8°C.

Sample Preparation

The internal standard (1000 ng/mL) was combined with 50 µL of the sample volume before being transferred into 100 µL polypropylene eppendorf tubes (ETs). About 3 mL of methyl tertiary butyl ether, mixed with stirring for about five minutes, was used to conduct the liquid-liquid extraction. The Eppendorf tubes were subjected to a 10-minute centrifugation run at 4000 rpm and 20°C. The supernatant was transferred to labeled polypropylene tubes and evaporated at 40°C using nitrogen gas. The samples were reconstituted with a solution of acetonitrile, 0.1% formic acid, and ammonium acetate (90:10), agitated for two minutes, and transferred to auto sampler vials for injection into the HPLC system coupled to the mass spectrometer.

Method Validation

The validation procedure was validated in accordance with the standards set out by the USFDA.[15-19]

Selectivity

In order to determine the level of selectivity of the technique, we subjected blank plasma samples from six healthy people to pre-treatment and testing. The purpose was to investigate whether any naturally occurring compounds in the samples may potentially disrupt the elution of the analyte and internal standard. The retention times and multiple-reaction-monitoring (MRM) responses were employed to identify and differentiate the chromatographic peaks of the analytes and internal standards. It is essential to ensure that the blank samples of pralsetinib and erlotinib do not exceed 20% of the average peak area at the limit of quantification (LoQ) for pralsetinib. Likewise, the erlotinib peak area in the blank sample should not surpass 5% of the average value.

Matrix effect

The absolute response of the reconstituted samples was compared to that of the quality control samples after pre-treatment using LLE with MTBE. This allowed us to quantify the ion enhancement/suppression in a signal by taking into account the matrix effect caused by plasma. The trials were performed in triplicate using a total of six unique plasma batches, all running at MQC levels. The permissible precision (%CV) was maintained at or below 15%.

Recovery

A total of six plasma samples, which were free from drugs, were artificially contaminated with equal amounts of pralsetinib and erlotinib. These spiked samples were then compared to six control samples of varying quality levels (low, medium, and high), with 6, 1100, and 1800 ng/mL concentrations, respectively. Our objective was to assess the extent to which pralsetinib and erlotinib could be detected and recovered in the samples.

We estimated the recovery of erlotinib by comparison of the mean peak areas of 6 quality control samples that were extracted, with samples produced by adding the same dose of erlotinib to extracted drug-free plasma samples.

Limit of detection and quantification

Limit of detection(LoD) is an example of such a metric that indicates a minimum amount in the sample which can be identified in relation to background noise, but cannot be precisely measured. The limit of detection (LoD) was estimated using a signal-to-noise ratio (S/N) of 3:1.The LoQ is the minimum concentration at which both precision and accuracy can be maintained while determining the quantity of an analyte. The LoQ was obtained by comparing known quantities of pralsetinib in plasma with mobile phase standards.

Calibration curve standards, regression model, precision and accuracy batches

Calibration curves were generated with pralsetinib concen-

trations ranging from 2.0 to 2000.0 ng/mL in plasma. The calibration curves were derived using weighted linear regression, with a weighing factor of 1/x^2, and a correlation coefficient exceeding 0.9997. This graph (Figure 2) illustrates the correlation between the pralsetinib/erlotinib ratio and the concentration of pralsetinib, measured in ng/mL. There were six distinct preparations of standards and quality control samples. The concentrations computed from the calibration sites in the past must have a reliability and exactness that falls within a range of ± 15% of their reported values. The lower limit of quantification (LLoQ) should have a accuracy and precision that is within a range of ± 20%.

Stability (Freeze-thaw, auto sampler, bench top, long term)

After three freeze-thaw cycles, samples were removed from a deep freezer in accordance with the clinical protocol. On a 24, 36, and 48-hour cycle, samples were kept at temperatures ranging from -10 to -30ºC. Furthermore, pralsetinib's longterm stability in quality control samples was evaluated for seventy-one days of storage at temperatures ranging from 10 to 30ºC. After being stored in the auto sampler tray for 55.5 hours, the stability of the auto sampler was tested at a chilled temperature. The stability of the bench top was examined over a 48-hour period. The stability samples need to be accurate to within 5% and within a minus fifteen percent range of their stated concentrations.

RESULTS AND DISCUSSION

Method Development and Validation

This study was done to develop and verify an assay method for accurately measuring the amount of pralsetinib in plasma samples. The method should be straightforward, efficient, and capable of detecting low concentrations of the compound. LCMS/MS has been widely used in clinical pharmacokinetics because of its capacity to selectively detect and measure substances, its high sensitivity, and its ability to produce consistent results. In order to enhance the performance of the mass spectrometer, the solutions of pralsetinib and erlotinibin were injected directly into the electrospray ionization (ESI) source of the instrument. The spray shape and ionization of protonated pralsetinib and erlotinib molecules were enhanced through the optimization of gas parameters, as well as ionization type, temperature, and voltage (Figures 3 and 4).

To enhance the resolution and amplify the signal of both the analyte and internal standard, a series of chromatographic settings were meticulously tuned through numerous iterations. These adjustments included optimizing the mobile phase composition and selecting an appropriate column. The extraction of olanzapine, pralsetinib, and erlotinib from the plasma sample involved the use of different optimization approaches, such as liquid-liquid-extraction (LLE), SPE(solid phase extraction) and precipitation procedures. The mobile phase, comprising a mixture of 5 mM ammonium formate and acetonitrile in a ratio of 10:90 v/v, facilitated efficient separation and elution. Flow rate of 0.6 mL/minute was consistently held, with an injection volume of 20 µL. In order to optimize the

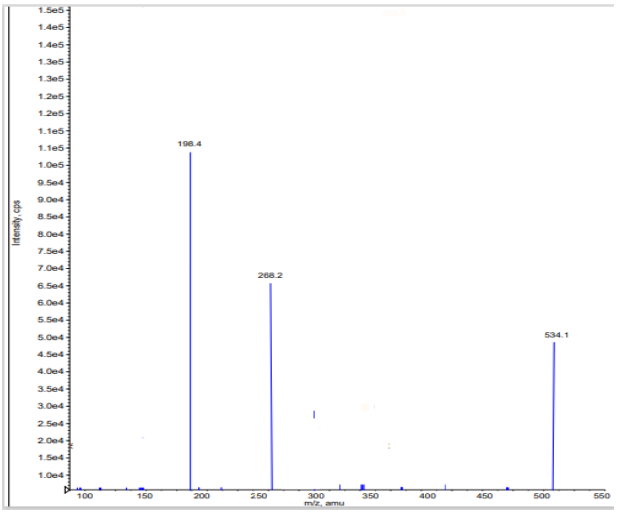

Figure 2: Mass spectra of pralsetinib (Parent ion)

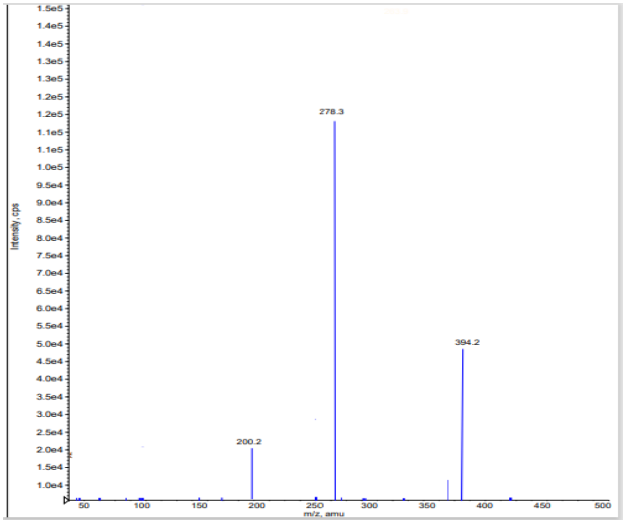

Figure 3: Mass spectra of erlotinib (Product ion)

effectiveness of the medication and the internal regulation, the method of liquid-liquid extraction was chosen. The compounds pralsetinib and erlotinib exhibited retention times of 0.94 and 0.93 minutes, respectively, as depicted in Figure 5.

Selectivity and Specificity

A comparison of chromatograms of blank plasma was used to evaluate the selectivity of the procedure. Both pralsetinib and erlotinib did not show any notable endogenous peaks at their respective retention times. The approach demonstrated high levels of selectivity and specificity, according to the results.

Linearity, Precision and Accuracy

Peak area ratio (pralsetinib/erlotinib) versus concentration (pralsetinib) was used to plot the calibration curve. Across the range of 2 to 2000 ng/mL concentrations, pralsetinib, the calibration was linear. In Table 1, we can see that for every curve, the correlation coefficient (r2) was higher than 0.9995. To ensure that this procedure was accurate and precise, the between-run and within-run variances of QC samples at 4 different concentrations (2, 6, 1100 and 1800 ng/mL) in 6

Table 1: Linearity data of pralsetinib

Spiked plasma conc. (ng/mL)	*Pralsetinib*	
	Concentration obtained (mean, ng/mL, n = 5)	*Peak area ratio*
2	1.99	0
4	4.19	0.01
8	8.16	0.01
20	20.15	0.02
40	40.1	0.04
80	80.42	0.12
100	101.63	0.23
500	497.79	0.31
1000	999.74	1.41
2000	1974.2	3.1

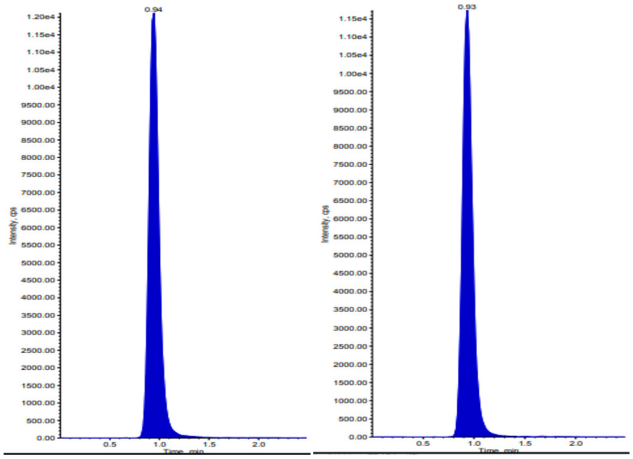

Figure 4: Standard chromatograms of a) Pralsetinib and b) Erlotinib

duplicates were calculated. Table 2 shows that the within-run precision ranged from 96.40 to 108.28% and the accuracy from 0.45 to 3.44. Accuracy ranged from 98.11 to 100.97% and between-run precision from 0.75 to 5.79%. Within the analytical range, our data show that, the approach is consistent and reproducible enough.

Matrix Effect

The signal at LQC and HQC levels showed ion suppression/enhancement of %CV 0.15 and 0.04, respectively. These findings suggest that the matrix effect on analyte ionization is not readily apparent in these settings.

Stability (Freeze-thaw, Auto sampler, Bench top, long term)

Three freeze-thaw cycles were performed on the plasma containing pralsetinib, ranging from -30°C to room temperature. Between 99.12 and 100.83% accuracy was achieved with pralsetinib. The accuracy of pralsetinib ranged from 99.55 to 100.85%, and there was no discernible deterioration of the drug even after 78 hours of storage in the auto sampler tray. On top

Table 2: Results of precision and accuracy

Compound name	*Spiked plasma conc. (ng/mL)*	*Within-run (n = 6)*			*Between-run (n = 30)*		
		Conc. measured (ng/mL, mean)	*Precision (CV, %)*	*Accuracy (%)*	*Conc. measured (ng/mL, mean)*	*Precision (CV, %)*	*Accuracy (%)*
Pralsetinib	6.0	6.13	0.72	102.13	6.07	0.75	101.13
	1100.0	1191.07	2.96	108.28	1163.07	3.03	105.73
	1800.0	1735.26	3.44	96.40	1977.26	5.79	109.87

Table 3: Stability of pralsetinib in plasma samples.

Stability experiments	*Spiked plasma conc. (ng/mL)*	*Pralsetinib*		
		Conc. obtained (n = 6, mean ± SD)	*CV (n = 6)*	*Accuracy (%)*
Benchtop (72 hours)	6.0	6.03 ± 0.00905	0.15	100.44
	1800.0	1803.57 ± 1.87	0.10	100.30
Autosampler (78 hours)	6.0	6.05 ± 0.5	0.15	100.75
	1800.0	1807.19 ± 8.26	0.46	100.85
Freeze–thaw stability (−30°C, cycle-3)	6.0	6.05 ± 0.01	0.25	100.83
	1800.0	1784.09 ± 28.33	1.59	99.12
Long-term stability (−30°C for 71 days)	6.0	6.05 ± 0.03	0.53	100.86
	1800.0	1787.40 ± 4.98	0.28	99.30

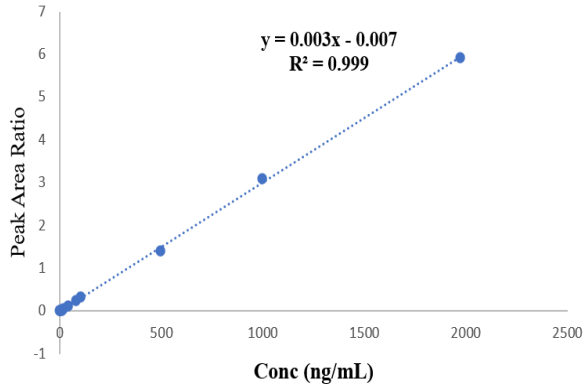

Figure 5: Calibration curve of pralsetinib

of that, we tested pralsetinib's room temperature stability for 72 hours and its long-term stability in QC samples stored at -30°C for 71 days. Results for stability experiments at room temperature ranged from 99.90 to 100.44%, while those for long-term stability varied from 99.04 to 101.50%. Based on these findings, pralsetinib remains stable in human plasma for a minimum of seventy-one days when stored at -30°C (Table 3).

Recovery

The pralsetinib extraction recoveries were 110.80, 100.03, and 100.61% at 6, 1100, and 1800 ng/mL, respectively. The pralsetinib and erlotinib overall average recoveries were 103.82 and 94.14%, respectively. Analyte and IS (internal standard) recoveries are repeatable, accurate, and consistent.

Limits of Detection and Quantification

The LoQ signal-to-noise (S/N) values found for six injections of pralsetinib at LoQ concentration was 9.8 pg/mL and LoD was 3.2 pg/mL.

CONCLUSION

With the application of tandem mass spectrometry's inherent selectivity, the assay method outperforms other approaches discussed before and is quite specific. We used LLE to extract the analyte. Several analytical parameters, such as linearity range, mobile phase, flow rate, injection volume, and plasma utilization volume, were optimized. Consequently, this method provides significantly improved selectivity, sensitivity, linearity, and reproducibility compared to previously reported approaches.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to the IICT in Hyderabad, India, for their assistance in providing data for this study. The authors are also thankful to the management of Chebrolu Hanumaiah Institute of Pharmaceutical Sciences for their constant encouragement.

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