AN LC–ESI-MS/MS METHOD DEVELOPMENTAND ITS VALIDATIONFOR THE QUANTIFICATION OF PRALSETINIB IN K2EDTA HUMANPLASMA

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ABSTRACT

Objective: Aim of the work is to developa simple LC-ESI-MS/MS approach for a sensitive, specific, and quantitative determination of pralsetinib in human K2EDTA plasma.

Methods: The samples were separated using an isocratic mobile phase made up of 0.1%HCOOH, methanol and acetonitrile in 10:70:20(% v/v) fractions. In multiple reaction monitoring mode, mass transitions for pralsetinib were m/z 534.27/302.19 and for asciminib internal standard, they were 450.1/257.10 in positive ionization mode.

Results: Linearity and a high r^2 value (>0.99) characterize the calibration curve, which spans 141.5–5660 ng/ml. Theintra-batchaccuracywaswithin-8.44 to 5.69% relative errorandtheprecisionwaswithin3.14 to 4.92%. Theinter-batchaccuracywaswithin-5.26 to 4.63% relative error and the precision was within 3.32 to 4.93%. The extraction recoveries executed at three quality control levels and their mean recovery was 97.45%.

Conclusion: In conclusion, a new approach to evaluating pralsetinib in human K2EDTA plasma has been approved for use in clinical, bioavailability, and bioequivalence investigations, and it may be used to quantify pralsetinib.

**Keywords:** Pralsetinib, Cancer, LC-MS/MS, K2EDT aplasma, Validation

INTRODUCTION

Pralsetinib is approved for treating metastatic non-small cell lung cancer (NSCLC) in adult patients with a confirmed rearranged during transfection (RET) gene fusion, as identified by an FDA-approved test. This treatment is also indicated for adult and pediatric patients aged 12 y and older who are dealing with advanced or metastatic RET fusion-positive thyroid cancer, particularly for those in need of systemic therapy when radioactive iodine is not suitable. The approval for advanced or metastatic RET fusion-positive thyroid cancer was granted under accelerated conditions, relying on the overall response rate and the duration of that response. Continued approval for this indication may depend on the verification and demonstration of clinical benefit in confirmatory trials [1, 2].

Rearranged during transfection (RET) is a type of protein called a receptor tyrosine kinase. It has parts that extend outside the cell, cross the cell membrane, and are inside the cell. RET is important for the right growth of the kidney and nervous system. Constitutive activation of RET happens via chromosomal rearrangements that form 5' fusions of dimerizable domains to the 3' RET tyrosine kinase domain, resulting in ongoing dimerization and autophosphorylation. The most common fusions are KIF5B-RET and CCDC6-RET, and more than 35 genes have been found to fuse with RET. Constitutive stimulation results in heightened downstream signaling and is linked to tumour spread, migration, and proliferation [3].

Pralsetinib molecular weight and chemical formula (fig. 1) are 533.612 g•mol−1 and N-[(1S)-1-[6-(4-fluoropyrazol-1-yl)pyridin-3-yl]ethyl]-1-methoxy-4-[4-methyl-6-[(5-methyl-1H-pyrazol-3-yl)amino]pyrimidin-2-yl]cyclohexane-1-carboxamide [4-6].

There have been no studies reported that use HPLC with tandem mass spectrometry (LC-MSMS) to measure pralsetinib by itself in human K2EDTA plasma. There are two ways reported for using LC-MS/MS with other drugs in rat plasma. The main goal of this study was to establish and test an efficient, accurate method using LC-MSMS to measure pralsetinib in K2EDTA human plasma.

Fig. 1: A) pralsetinib and B) Asciminib chemical structures
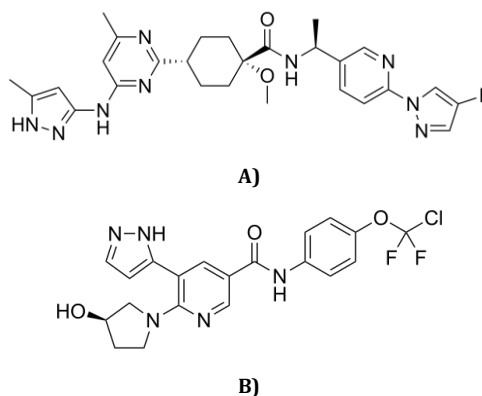
MATERIALS AND METHODS

Reagent chemicals

The pure pralsetiniband asciminib standards came from SoluClinipharm Private Limited, in Mumbai, India. Acetonitrile and formic acid that are suitable for HPLC were provided by Finar Chemicals in Ahmedabad, India. HPLC grade water (Millipore, USA) was made by filtering it through a Millipore MilliQ plus device.

Instrument

Quantum Discovery TSQ mass spectrometryanalyzer (Thermo Finnigans, Sans Jose, C. A, USA) with an electrospray ionization interface was used for chromatography. It had an auto sampling and degassing system, and pumps connected to it. The test was done on a Zorbax C18 (50 × 2.1 mm i. d., 3μm particles size) reversed phase column. The Xcalibur program (version 1.4) was used to handle all the tasks, including gathering and analyzingdata.

Mass and liquid chromatographic system conditions

The samples were separated using an isocratic mobile phase consisting of 0.1%HCOOH, methanol, and acetonitrile in 10:70:20(% v/v/v) fractions. The column was maintained at a temperature of 30 °C and a flow rate of 0.5 ml/min. The spray voltage was set to 4500V, the vaporizer temperature to 250 °C, and the capillary temperature to 350 °C in order to monitor multiple reactions. pralsetinib's optimal MS transitions in MRM mode were m/z 534.27/302.19, while asciminib 's were 450.1/257.10 in positive ionization mode.

Processing of calibration, quality control and standard solutions

The IS (1000μg/ml) and the stock solution of pralsetinib (1000μg/ml) were processed individually in methanol and subsequently monitored at 5 °C in a refrigerating system [7-13]. In order to process the standards for working controls for pralsetinib and 250 ng/ml for asciminib (IS), appropriate dilutions were prepared in mobile phase. The standard controls for pralsetinib and asciminib were executed when required and retained in a refrigerator at 2 to 8 °C.

Linearity control solutions of pralsetinib were processed by introducing 1% quantities of control solutions into plasma blank. The calibration curve was determined to have eight levels. The plasma concentrations of pralsetinib were 141.5, 450, 900, 1600, 2400, 3400, 4500 and 5660 ng/ml at the end of the study [14-19]. The quality control (QC) levels were similarly executed with plasma blank to get 141.5ng/ml, 396.2 ng/ml, 2830ng/ml, and 4245ng/ml for the LLOQ, LQC, MQC, and HQC, respectively. The plasma sample preparation procedure was followed for all contaminated samples.

Processing of sample solution

50μL aliquots of each linearity level, Q. C sample solutions, and test solutions were combined with 150μl of aninternal working standard solution (1μg/ml) and shaken vigorously using a vortexer [16-22]. Following vortex mixing, every solution was rapidly cooled by adding 800μl of methanol and subjected to mixing for 30 sec. Mixture was then subjected to centrifugation at 15,000 revolutions per minute for at least 25 min at a temperature of 4 °C. Following centrifugation, the liquid portion above the sediment was meticulously isolated, and 5μl portion was introduced into an LC–ESI-MSMS instrument for quantification.

Validation of the analytical method

Developed an LC–ESI-MSMS technique to assess pralsetinib from biological matrices was validated as per the U. S. FDA bioanalytical method validation guidelines (2018) [7-15]. Developed procedure was subjected for validation for the parameters selectivity, accuracy, linearity, specificity, matrix effect, extraction recoveries, precision, carryover, dilution integrity and stability.

Selectivity

The evaluation of selectivity was conducted using the K2EDTA plasma without any substances present. The assessment of selectivity should indicate that there were no notable interferences detected for IS and pralsetinib retention times in blank plasma [9-12].

Calibration plot and sensitivity

Linearity was detected across eight calibration curves spanning a concentration range of 141.5ng/ml to 5660ng/ml for pralsetinib. The linerarityplots were generated by graphing peak response fractions of pralsetinib to asciminib against the control solutions of linearity controls [10].

Accuracy and precisions

To ensure method's precision and accuracy, every QC level was tested 6 times in 3 separate batches, both intra-day and inter-day. Precision and accuracies findings within a range of ±15% were deemed acceptable, with the exception of concentrations at LLOQ, for which a tolerance of 20% was deemed acceptable [11].

Matrix effect and extraction recoveries

The extraction recoveries was assessed by computing the fraction of response of QC levels containing known quantities of pralsetinib to response of pralsetinib introduced at similar quantities to blank plasma after protein precipitation. This analysis was performed three times (n = 3) [12, 13]. The matrix impact was assessed by comparing the quantities of pralsetinib dissolved into matrix blank extract to those of pure standards (n = 3).

Dilution linearity

Dilution integrity refers to the evaluation of diluting process of the sample to ensure that it does not affect precision and accuracies of assessed concentrations of pralsetinib. The solutions diluted were tested and their precision and accuracies were evaluated to expected findings. Results that fell within a range of ±15% were deemed to be satisfactory in terms of both accuracy and precision [14-17].

Carryover

The carryover was evaluated by measuring peak responses of pralsetinib by injection of blank matrix samples in a sequential manner after the injection of the highest calibration standard sample [16, 19].

Stability

The stability of the human K2EDTA plasma samples was examined by assessing them at two distinct concentration levels of quality control (QC) samples. This analysis was conducted under various settings, comparing the samples against recently created calibration curves and QC samples. The concentrations of the QC samples were 396.2 and 4245 ng/ml, with a total of 6 samples analyzed. The plasma samples underwent storage for a minimum of 6 h at room temperature, at a temperature of-20 °C for ten days, 3 cycles of freezing and thawing, and for 24h at a temperature of 4 °C in auto sampler [19-21].

RESULTS AND DISCUSSION

Validation of the analytical method

Selectivity

Fig. 2 and 3 demonstrate that there was no interference detected in retaining times of pralsetinib (0.83 min) and asciminib (1.14 min) in chromatograms of plasma blank solutions taken from different 6 batches. Findings demonstrated that pralsetinib test exhibited a high degree of specificity and selectivity.

Calibration plot and sensitivity of pralsetinib

Linearity controls were executed and examined at eight distinct concentration levels ranging from 141.5ng/ml to 5660ng/ml. Linearity graph for pralsetinib exhibited satisfactory linearity throughout the specified concentration range, as shown by a mean correlation coefficient (r2) greater than 0.99 [21-26]. The curve was weighted using a factor of $1/x^2$. The equation for linear regression is y = 0.000442x-0.00665, as shown in table 1. These calculated concentrations fell within the acceptable range of ±15%, except for the LLOQ, where the acceptable range was ±20%.
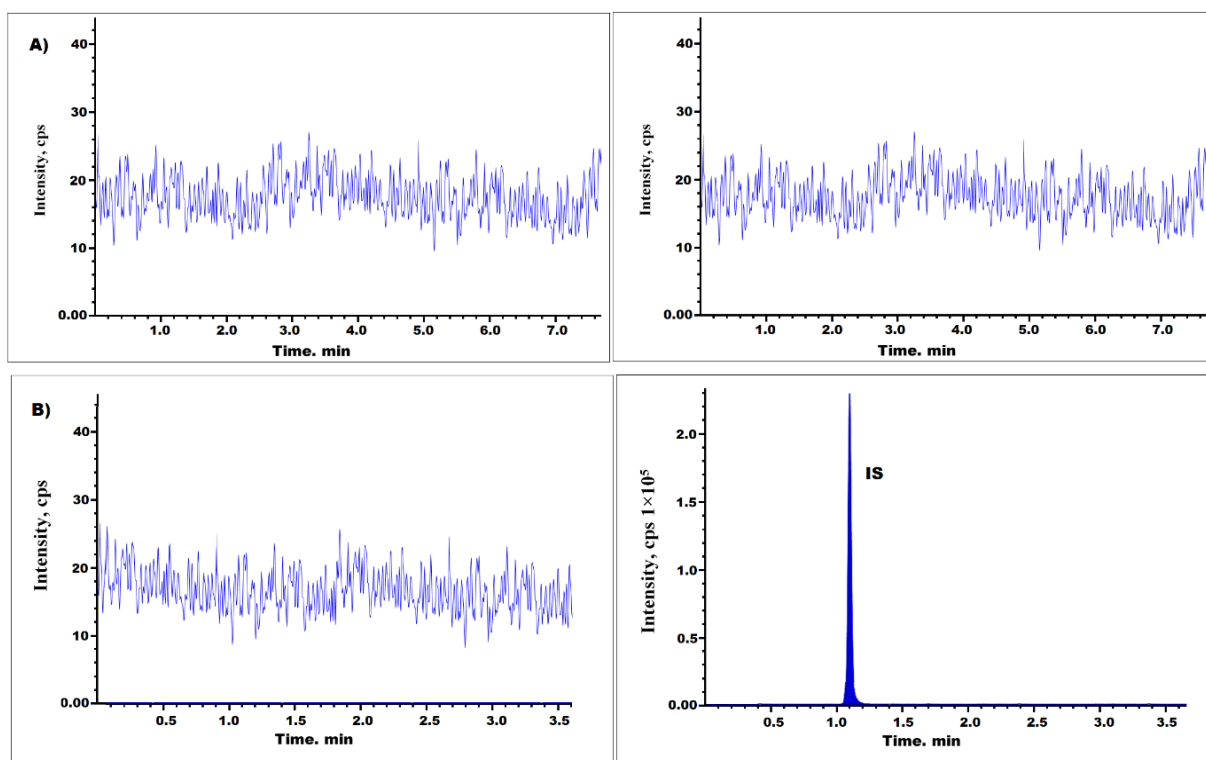
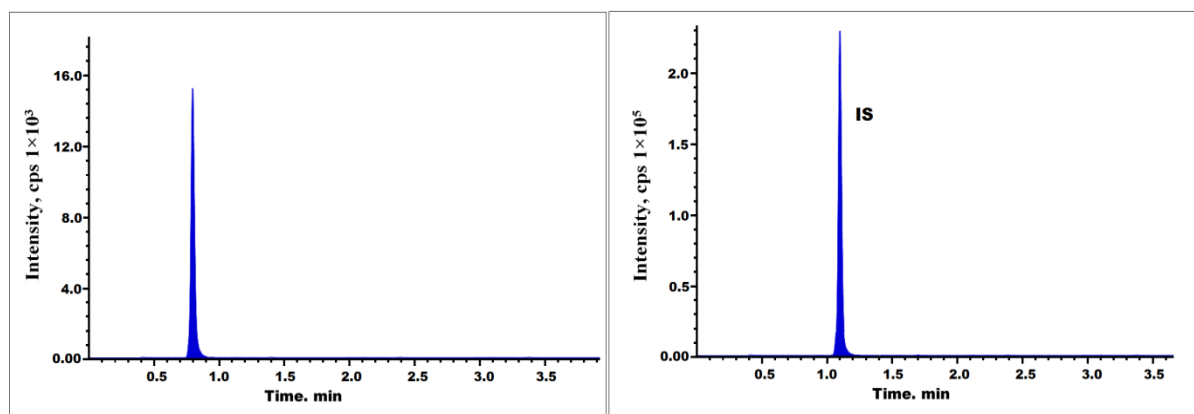**Fig. 2: Pralsetinib chromatograms at blank plasma (A), and blank plasma with IS (B)**
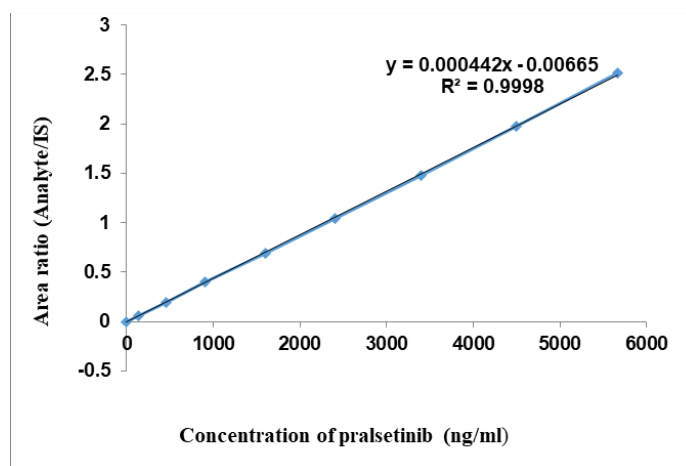

Fig. 3: pralsetinib chromatogram at LLOQ level

$y = 0.000442x - 0.00665$
$R^2 = 0.9998$

Fig. 4: Linearity of pralsetinib

Table 1: Pralsetinib linearity data

LS-ID	Concentrations in ng/ml	Mean^n area	IS area	Analyte/IS area
LS1	141.5	15025	243364	0.061739
LS2	450	47782	243951	0.195867
LS3	900	96165	243457	0.394998
LS4	1600	168893	243749	0.692897
LS5	2400	254740	243634	1.045585
LS6	3400	360824	243368	1.482627
LS7	4500	479026	242813	1.972819
LS8	5660	611846	242994	2.517947

LS: Linearity standard; n=6number of replicates.

Precision and accuracies

The intra-batchaccuracy was within 8.44 to 5.69% relative error and the precision was within 3.14 to 4.92%. Theinter-batchaccuracywaswithin-5.26 to 4.63% relative error and the precision was within 3.32 to 4.93%. The assay results, as shown in table 2, were below 15% and met the approved limit. This indicates that the quantification of pralsetinib in human K2EDTA plasma was accurate and precise.
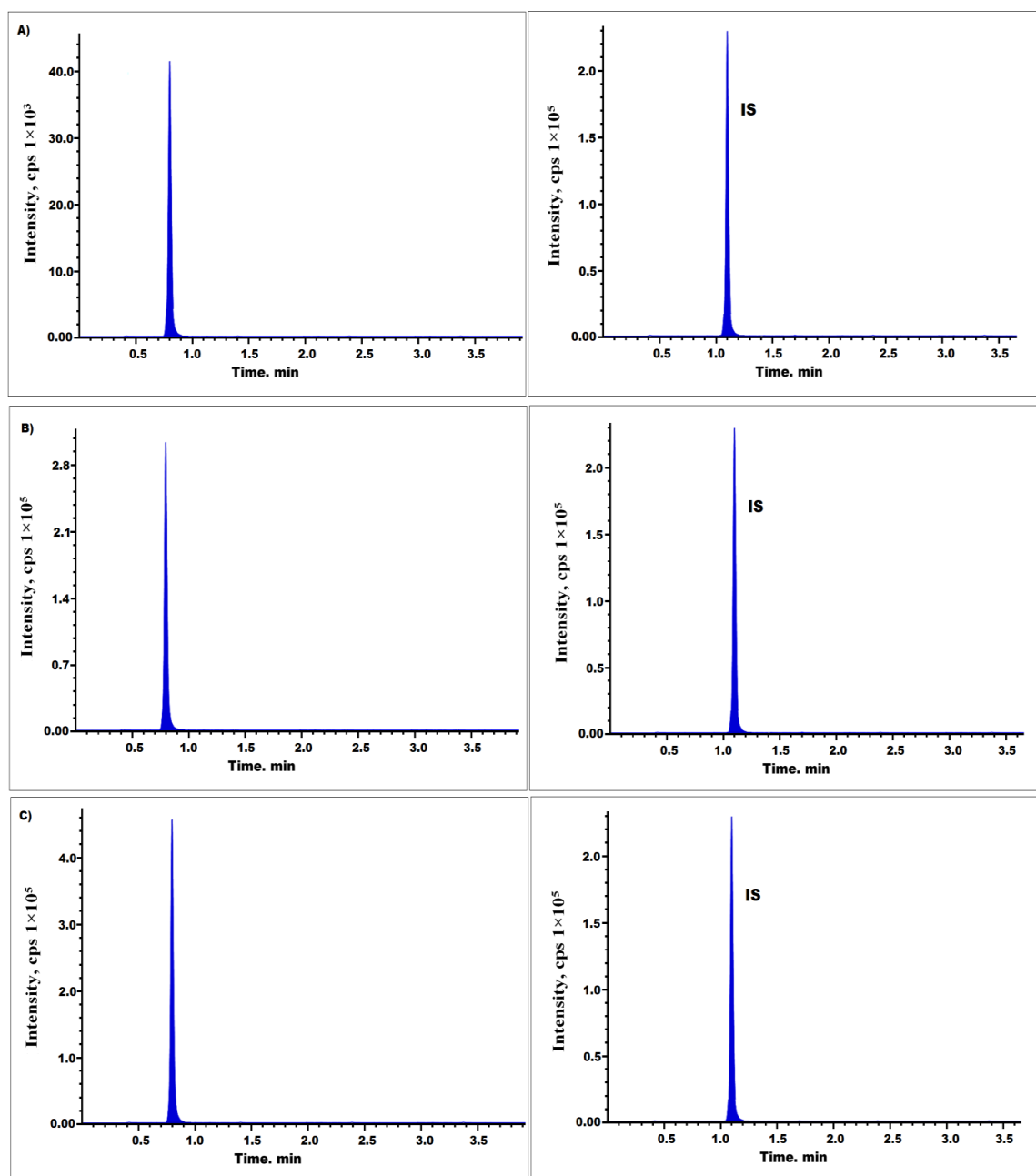

Fig. 5: Pralsetinib chromatograms at LQC (A), MQC (B), and HQC (C) levels

Table 2: Intraday and interday precision and accuracies of pralsetinib

Level of drug spiked (ng/ml)		Intra-day[a]			Inter-day[b]		
		Concentrations found (mean±SD; ng/ml)	Precision (%RSD)	Accuracy (% RE)	Concentration found (mean±SD; ng/ml)	Precision (% RSD)	Accuracy (%RE)
Pralsetinib	141.5	139.06±5.35	3.85	-1.73	148.06±4.93	3.33	4.63
	396.2	418.76±15.87	3.79	5.69	408.76±16.32	3.99	3.17
	2830	2591.15±127.38	4.92	-8.44	131.81±131.81	4.93	-5.26
	4245	4024.73±126.23	3.14	-5.19	136.24±136.24	3.32	-3.19

a: 6 number of replicates; b: 18 number of replicates.

Matrix effects and extraction recoveries

The analysis of extraction recoveries (table 3 and fig. 5) and matrix effect revealed that the extraction recoveries for pralsetinib satisfied the specified parameters, and the matrix impact in this assay was found to be insignificant (table 4).

Dilution integrity

Six duplicate samples, each containing pralsetinib at a concentration of 6 µg/ml, were diluted by factors of 5 and 10 using blank plasma. The analyte exhibited a precision (%CV) of less than 15% and an accuracies within 85-115%.

Carryover

Within the scope of this investigation, the blank matrix samples were evaluated in a sequential manner following the highest linearity concentration, and there was no evidence of a carryover effects.

Stability

Pralsetinib demonstrated stability in human K2EDTA plasma under the following temperature and time conditions: a minimum of 6 h at room temperature, storage at-20 °C for 10 d, exposure to three freeze-thaw cycles, and stored at 4 °C for 24 h in auto sampler. The stability test results are shown in table 5.

Table 3: Pralsetinib extraction recoveries

QC level	X	Y	% Recoveries	% mean recoveries	%RSD
LQC	42216	39602	93.81	97.45	4.34
MQC	300583	286034	95.16		
HQC	450763	465953	103.37		
IS	243179	238169	97.94		

X, average recoveries of (n=3) unextracted samples; Y, average (n=3) recoveries of extracted samples

Table 4: Pralsetinib matrix effect at LQC and HQC levels

S. No.	LQC			HQC		
	Peak responses in absence of matrices	Peak responses in existence of matrices	Matrix factor	Peak responses in lack of matrices	Peak areas in presence of matrices	Matrix factors
1	42147	39833	94.51	453148	430944	95.1
2	42364	40686	96.04	452753	467558	103.27
3	42741	43749	102.36	453617	431299	95.08
4	42073	39468	93.81	452581	423706	93.62
5	42259	39081	92.48	453624	470453	103.71
6	42627	44464	104.31	451963	425116	94.06
Mean±SD			97.25±4.89			97.47±4.69
% RSD			5.03			4.82

Table 5: Stability findings of anaytes (n = 3)

Storage temperature	LQC		HQC	
	%Accuracy	Precision	%Accuracy	Precision
6 h at ambient temperature	95.28	4.01	95.32	2.84
4 °C in auto sampler	94.37	3.91	98.07	3.61
3 freeze-thaw cycles	97.01	2.87	93.64	4.37
−20 °C for 10 d	93.27	4.31	92.57	4.09

Recently one method was reported on UPLC-MS/MS for the determination of pralsetinib in Human Plasma and Cerebrospinal Fluid [27] using ACQUITY UPLC HSS T3 column (2.1×100 mm, 1.8 µm) with a gradient elution using 0.1% formic acid (solution A) and acetonitrile (solution B) as mobile phases at a flow rate of 0.4 ml/min. In the present method chromatography was executed on a Zorbax C18 (50 × 2.1 mm i. d., 3µm particles size) reversed phase column with an isocratic mobile phase consisting of 0.1%HCOOH, methanol, and acetonitrile in 10:70:20(% v/v/v) fractions. In the reported method a linear relationship of pralsetinib in both plasma and CSF was successfully established, and the calibration ranges were found to be 1.0-64.0 µg/ml and 50.0ng/ml-12.8 µg/ml for pralsetinib in the plasma and CSF, respectively. In the current method linearity was detected across eight calibration curves spanning a concentration range of 141.5ng/ml to 5660ng/ml for pralsetinib. The equation for linear regression is y = 0.000442x-0.00665.

CONCLUSION

Aim of the work is to present a simple LC-ESI-MS/MS approach for a sensitive, specific, and quantitative determination of pralsetinib in human K2EDTA plasma. The samples were separated using an

isocratic mobile phase made up of 0.1%HCOOH, methanol and acetonitrile in 10:70:20 (% v/v) fractions. In multiple reaction monitoring mode, mass transitions for pralsetinib were m/z 534.27/302.19 and for asciminib internal standard, they were 450.1/257.10 in positive ionization mode. Linearity and a high r^2 value (>0.99) characterize the calibration curve, which spans 141.5–5660 ng/ml. The intra-batch accuracy was with in-8.44 to 5.69% relative error and the precision was within 3.14 to 4.92%. The inter-batch accuracy was with in-5.26 to 4.63% relative error and the precision was within 3.32 to 4.93%. The extraction recoveries executed at three quality control levels and their mean recovery was 97.45%. As per the bioanalytical technique validation guideline, the LC-MS/MS method was validated in terms of accuracy, linearity, precision, specificity, stability, dilution integrity, extraction recoveries,

AUTHORS OF CONTRIBUTIONS

Sunil Dattatray Pawar performed the research work and prepared the manuscript. Deepak D Kayande, Kokila Pawar, Mazahar Farooqui, Anil Shankar war, Amol Deshpande, and Sunil Shankar war were corrected the manuscript.

CONFLICT OF INTERESTS

Declared none

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