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Quantification of Alectinib in spiked rabbit plasma using liquid chromatography- electro spray ionization-tandem mass spectrophotometry: An application to pharmacokinetic study

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A B S T R A C T

The current technique was developed to estimate the amount of alectinib present in spiked rabbit plasma using liquid chromatographic mass spectrometry. The liquid-liquid extraction method was used, and chromatographic separation was carried out on a C18 (4.6mm id x 50mm) analytical column with a mobile phase consisting of acetonitrile and water with 0.1% formic acid at a volume ratio of 75:25. Alectinib's product m/z +483.2 (parent) 396.1 (product) and the internal standard m/z +447.5 (parent) 380.3 (product) were both obtained using positive ion mode. The calibration curve was linear from 0.5 to 600 ng/ml. The percentage extraction recovery (98.15% → 98.86%), demonstrated excellent matrix and analyte selectivity (% interference = 0), and satisfactory stability study results in all types (% nominal 94.94% → 99.63%). The intra and interday accuracy with % nominal 97 → 98.8%, precision % CV ≤ 2% in all quality control levels. The rabbit model's pharmacokinetic parameters were examined, and alectinib's area under the curve (AUC 0—∞) was 4269 ± 8.13 hr.ng/ml. The half-life of elimination (t1/2) is 8.52 ± 6.66 hours. The currently established approach was used in rabbit blood samples for pharmacokinetic investigations of commercial formulations since it was thought to be a novel, verified bioanalytical method based on experimental results.

1. Introduction

One type of cancer that begins in the lungs is lung cancer. When body cells start to proliferate uncontrollably, cancer begins to develop. NSCLC (Non-small cell lung cancer) accounts for 82–86% of lung cancer cases. Squamous cell carcinoma, adenocarcinoma, and giant cell carcinoma are the three main subtypes of NSCLC. Since their treatment and prognosis (outlook) are frequently comparable, these subtypes are classed together as NSCLC and originate from different types of lung cells[1]. The alectinib which is chemically is an organic heterotetracyclic molecule (See **Fig. 1**) with extra cyano, 4-(morpholin-4-yl)piperidin-1-yl, and ethyl substituents at positions 3, 8, and 9 respectively[2]. Alectinib is a tyrosine kinase receptor inhibitor of anaplastic lymphoma kinase (ALK) with anticancer potential. When taken, alectinib binds to and disables ALK kinase. The inhibition finally stops tumour cell proliferation in ALK-overexpressing tumour cells by causing disruption of ALK-mediated signaling[3]. In November 2017, the FDA approved alectinib (Alecensa), a TKI that targets both ALK and RET, for use as frontline therapy in patients with metastatic NSCLC that tests positive for ALK. The FDA has expanded the use of alectinib for patients with ALK-positive NSCLC who have progressed to crizotinib in addition to granting it this new indication[4,5].

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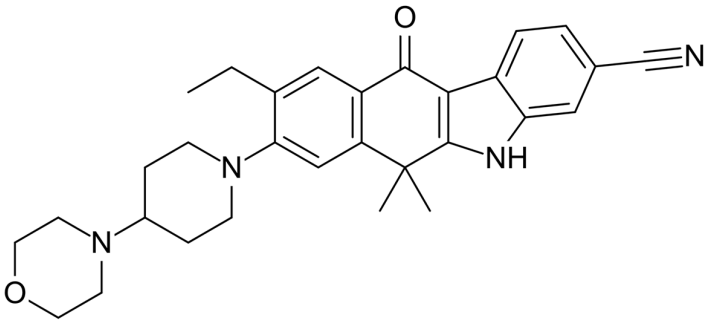

Fig. 1. Chemical structure of Palbociclib

Extensive literature review for the alacetinib estimation method using different analytical tools was conducted, it was disclosed that only two HPLC methods[6,7] one UPLC method[8], are available for the alectinib estimation in tablet dosage form. Two LC-MS methods[9,10] for the estimation of alectinib in human plasma and their pharmacokinetic study were also published. Heinig *et al.* in 2016 developed the method of bioanalysis of alectinib and metabolite M4 in human plasma, where they utilized protein precipitation method for the extraction of drug from the plasma. The Potential problems can occur in this technique, such as losses due to poor solubility of compounds in the extraction solvent, solvent saturation effects, analyte co-precipitation with proteins, and poor chromatographic separation due to co-elution with endogenous compounds such as phospholipids[11]. The utilized internal standard alectinib D8 is not easily available for routine work. They also performed a cross validation study, overall, the method looks complex. Another method developed by Huang *et al.* in 2017 utilized the same protein precipitation method for the extraction of drug, and lower limit of quantitation can be reduced from 2 ng/mL for the alectinib determination in rodent model. After considering the above disadvantages of the existing methods, there is a need to develop and validate a novel, easy and feasible bioanalytical method for the estimation of alectinib in plasma samples. Therefore, efforts were made with the goal of resolving the noted drawbacks mentioned above, developing and validating a novel sensitive LC-MS/MS method for alectinib alone in rabbit plasma in accordance with USFDA guidelines[12, 13], and applying the method created for the pharmacokinetic analysis using rabbit plasma samples for routine analysis, bioavailability study, and further clinical investigation in rabbit plasma[14]. Overall, in the present method, the more feasible liquid-liquid extraction method was utilized, which solved the problems of the previously reported methods, like co-precipitation, sample loss, complexity. The lower limit of quantitation has been reduced which increased the sensitivity directly in comparison to the previous method. Easily available and suitable internal standard has been used in the present method.

2. Results and Discussion

2.1 Mass spectrometry

In order to build the approach, the initial LC-MS/MS was specified by accurate tuning of all parameters. Table 1 provided a comprehensive list of the parameters. In positive ion mode, a straightforward parent ion was produced by alectinib and the internal standard palbociclib. The m/z of Alectinib was determined to be 483.2, while the m/z of Palbociclib was 447.5. The internal standard [M+H]+ ion was employed as a precursor ion to acquire Q3 product ion spectra, and the parent ion in the Q1 section was protonated alectinib.

Table 1. LC-MS/MS tuning parameters (Positive ion mode)
MRM= multiple reaction monitoring

Parameters	Alectinib	Internal standard (Palbociclib)
MRM	m/z +483.2 (parent) m/z + 396.1 (product)	m/z +447.5 (parent) m/z +380.3 (product)
Declustaring Potential DP (V)	72	72
Collision energy CE (V)	35	35
Collision exit cell potential (V)	26	32
Source temperature	300^0C	300^0C
Entrance potential	10 V	10 V
Ion spray voltage	5000V	4500V
Focusing potential	350 V	350V
Dwell scan	150 ms	150 ms
Desolvation temperature	700^0C	700^0C
Desolvation gas flow (L/h)	73	73
Curtain gas	12 PSI	12 PSI

2.2 Method development

The method was developed by a series of chromatographic tests using various mobile phases with variable volume ratios. Several mixtures of acetonitrile, methanol, and buffers were used in the initial research. The detected peaks of the analyte alectinib in the early tests were poor because of numerous splits and a lot of baseline noise. Finally, an analytical column with a water symmetry shield C-18 (4.6mm id x 50mm) was utilized. The mobile phase used was acetonitrile: water (0.1%) formic acid in a volume ratio of 75:25. The peak shapes for the internal standard and analyte were found to be excellent in this optimized condition, even at very low-quality control samples. Numerous chemicals were investigated in search of an appropriate internal standard. Palbociclib was ultimately selected because it had a very selective retention time and other parameters that did not interfere with analytes. According to the MRM chromatograms in **Fig. 2**, the internal standard's retention time at this optimized condition was determined to be 0.633 minutes, and the retention time for alectinib was 1.51 minutes.

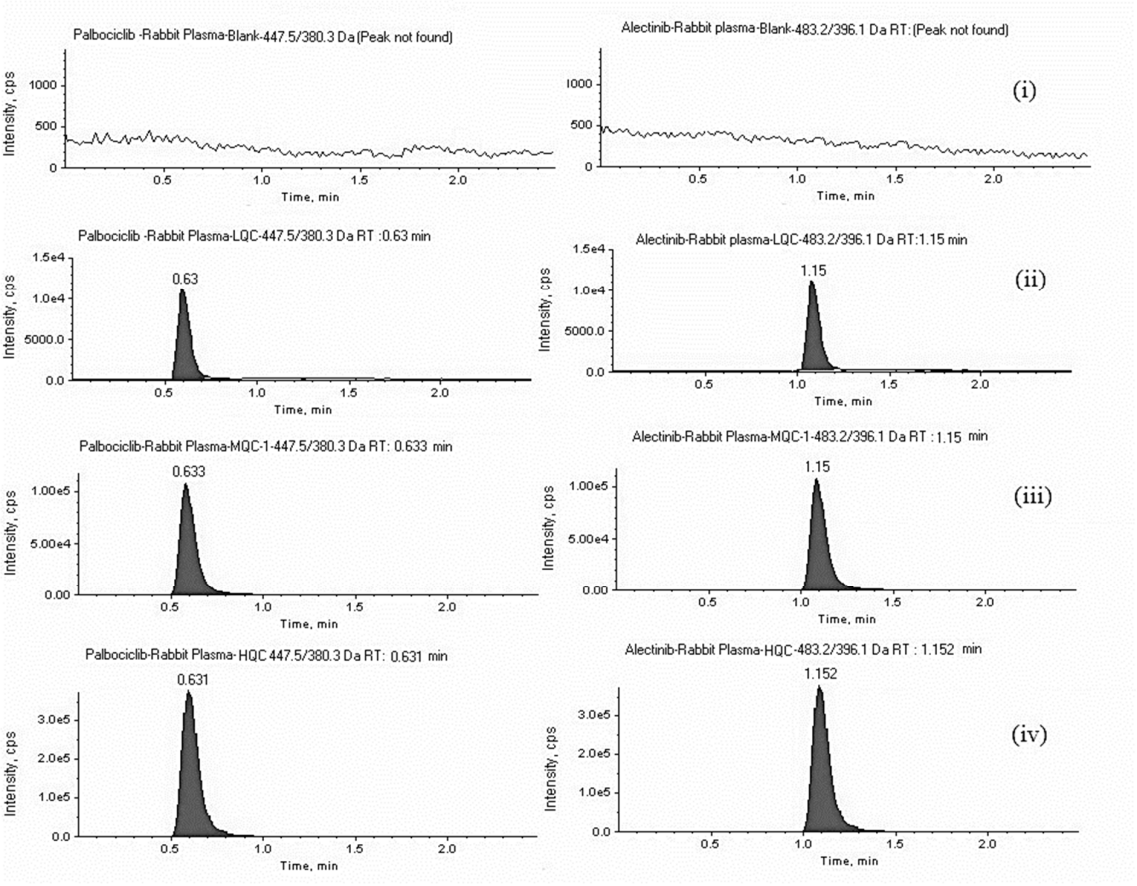

Fig. 2. MRM chromatograms of Alectinib, Blank plasma (i), LOQ samples with internal standard [right sequence] (ii), MQC-1 samples with internal Standard [right sequence] (iii), HQC samples with internal Standard [right sequence] (iv)

2.3. Validation

Several validation factors for alectinib were examined in accordance with USP standards using the established enhanced technique. The matrix effect led to the determination of the internal standard normalised factor for the alectinib in the presence of matrix ion. Because the percent CV of the Is normalised factor was found to be 1.24% for LQC samples and 0.89% for HQC samples, acceptance conditions were satisfied. No interference was found at the retention times of the analyte and internal standard, and the response of interfering peaks at the retention times of alectinib and palbociclib was found to be 0 % of the mean drug response when the mean response of extracted LLOQ samples was compared with the blank matrix. The carryover research findings show that there was no interference in the retention period of alectinib with the internal standard palbociclib at the ULOQ and LLOQ levels. The acquired results for the blank samples are zero and the computed percent carry over is zero as well, showing that the acceptance criteria were satisfied. In a study of intraday within batch precision (% CV), the intraday within batch precisions (% CV) of the alectinib LLOQ, LQC, MQC-I, MQC-II, and HQC samples were reported to be 2.65, 1.77, 1.61, 1.69, and 1.89, respectively. The intraday accuracy (% Nominal) for LLOQ, LQC, MQC-I, MQC-II, and HQC was 95.77%, 97.61%, 98.87%, 98.23%, and 97.35%, respectively. For the levels of LLOQ, LQC, MQC-I, MQC-II, and HQC, the batch accuracy (% Nominal) results were 96.42%, 98.28%, 98.38%, 98.86%, and 99.23%, while the precision (% CV) values were 1.76, 1.83, 1.59, 0.67, and 1.69. Table 2 highlights the

specifics of the results. For samples of the alectinib calibration curve in the concentration range of 0.5 to 600 ng/ml, a regression equation with a weight factor of 1/(concentration ratio)^2 of drugs to internal standard concentration was utilized in the linearity investigation. Alectinib's correlation coefficient (r^2) was found to be 0.998. With a precision (percent CV) range of 0.67 to 1.63 and a percent difference between height and lowest percent recovery of 4.33 for alectinib and 7.93 for palbociclib, respectively, respectively, the mean overall recovery of alectinib was found to be 96.77% and 96.56% for internal standard in a recovery analysis of Alectinib and internal standard QC samples. The outcomes fell within allowable limits. In this study, the dilution integrity was assessed at the ULOQ level. % CV and % nominal were determined to be 1.36 and 97.68% at two times dilution and 1.43% and 96.52% at four times dilution, respectively, in contrast to the undiluted calibration curve samples. For the ruggedness investigation, within batch accuracy (% CV) values were reported to be 1.16, 1.37, 0.67, 0.87, and 5.34. The accuracy percentages were 97.76%, 96.83%, 97.42%, 98.53%, and 92.16% for the LLOQ, LQC, MQC-I, MQC-II, and HQC levels.

Table 2. Accuracy and precision for determination of alectinib in rabbit plasma.

QC levels (ng/mL)	Alectinib measured concentration (ng/ml)				
	Run*	**Mean**	**SD**	**% CV**	**% Nominal**
	"Between the Batch intraday"				
LLOQ (0.5)	1	0.49	0.43	2.65	95.77
	2	0.48	0.45	1.67	97.68
	3	0.48	0.65	3.58	96.93
	4	0.48	0.89	2.86	88.43
LQC (10)	1	9.68	1.52	1.77	97.61
	2	9.56	0.37	1.67	90.83
	3	9.77	0.65	1.81	98.68
	4	9.79	1.88	1.48	97.93
MQC-1 (100)	1	98.93	1.39	1.61	98.87
	2	97.75	1.76	1.17	49.02
	3	99.05	1.68	1.38	98.67
	4	97.26	1.67	2.19	98.37
MQC-II (200)	1	193.57	1.55	1.89	97.35
	2	194.89	0.27	0.77	99.58
	3	196.86	1.64	1.17	98.72
	4	197.37	0.69	0.83	98.70
HQC (300)	1	291.74	4.29	1.73	98.23
	2	289.63	5.23	2.59	97.22
	3	289.46	7.78	1.68	97.49
	4	292.28	2.87	7.46	93.96
	"Within batch Interday"				
LLOQ		0.49	0.75	1.76	96.42
LQC		9.79	0.38	1.83	98.28
MQC-1		99.40	0.67	1.59	98.38
MQC-II		198.68	0.28	0.67	98.86
HQC		291.86	2.37	1.69	99.23

*each run includes six replicates.

2.4. Stability studies

The findings of a stock solution stability investigation at room temperature (20±5^{0}C) for alectinib and the internal standard were 100.58% and 99.63%, respectively. In the refrigerator stock solution stability investigation at 2-8^{0}C for 4 days, the internal standard and alectinib both had computed stability percentages of 96.24 and 98.24, respectively. The percent nominal for Bench top stability research results was 91.68% at the LQC level and 95.68% at the HQC level. According to the findings of a four-cycle freeze-thaw stability sample, alectinib has a broad range of acceptability at both the LQC and HQC levels, with percentage stability of 95.90 percent and 96.68 percent, respectively. At the LQC and HQC levels, respectively, the autosampler stability research (72 hours of acceptable stability time in autosampler) showed 98.67% and 93.21%. The nominal value for alectinib in wet extract stability testing was 97.85 percent for LQC and 96.84 percent for HQC. The computed percent stability for alectinib was greater than 93.80% at LQC and 98.87% at HQC, which was above the acceptable range for the short-term stability research. In the long-term stability tests, the percentage of stability for LQC samples was 96.68%, and for HQC samples it was 98.65%. (30 days at -70^{0}C). Table 3 compiles the results of all stability investigations.

Table 3. Stability study data of Alectinib

QC levels	Type of stability	Alectinib			
		Mean*	**SD**	**% CV**	**% Nominal**
LQC	Bench Top	9.08	0.17	1.89	91.68
	Freeze thaw	9.39	0.23	2.18	95.90
	Autosampler	9.32	0.29	2.86	98.67
	Wet extract	9.60	0.24	2.35	97.85
	Short term	9.86	0.20	2.78	93.80
	Long term	9.83	0.14	1.73	96.68
HQC	Bench Top	281.34	6.43	2.74	95.67
	Freeze thaw	287.89	5.99	1.67	96.68
	Autosampler	286.68	2.96	1.41	93.21
	Wet extract	289.07	8.98	1.67	96.85
	Short term	285.41	8.96	1.88	98.87
	Long term	279.04	3.23	1.42	98.65

*mean of six replicates

2.5. Result of pharmacokinetic study

The pharmacokinetic parameters of the kinetic research were computed using the non-compartmental model, and the area under the curve (AUC_{0}-∞) for alectinib was 4269 ± 8.13 hr.ng/ml. Elimination half-life ($t_{1/2}$) is 8.52 ± 6.66 hours. All of the various parameters are listed in detail in table 4 of the kinetic parameters. The plasma concentration and the time profile are shown in **Fig. 3. Fig. 4** displays the MRM chromatograms of alectinib during the kinetic analysis in rabbit blood samples.

Table 4. Pharmacokinetic parameters of Alectinib

Pharmacokinetic parameters	Palbociclib
C_{max} (ng/ml)	379.6 ± 2.51
t_{max}	7.13 hr ± 2.59
AUC $_{0-\infty}$ (h* ng/ml)	4269 ± 8.11
AUC $_{0-t}$ (h* ng/ml)	4366 ± 7.52
$t_{1/2}$ (h)	8.52 ± 6.66

AUC (Area under the curve),

Based on the obtained results it can be discussed that following fruitful tests to adjust the parameters for both chromatographic and mass spectrophotometric analysis, the current bioanalytical method for alectinib was created. Alectinib and the internal standard Palbociclib's production mass spectra were initially produced using optimized LCMS parameters. Acetonitrile and ultrapure water (0.1 percent formic acid), in the volume ratio of 75:25, have been used to optimize the MRM chromatograms of the drugs alectinib and palbociclib at the same tuning conditions.

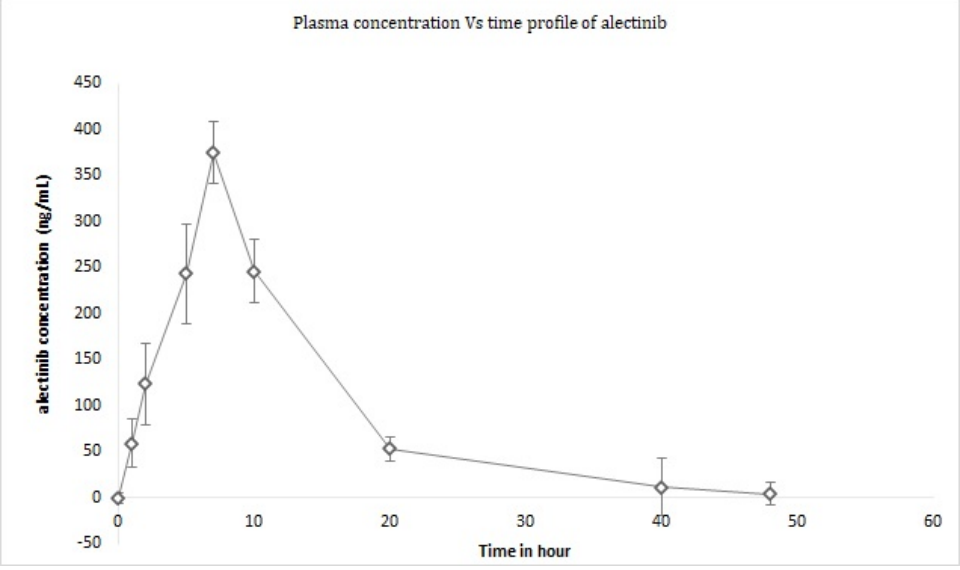

Fig. 3. Mean plasma concentration –time profile of alectinib after oral administration of tablet dosage form

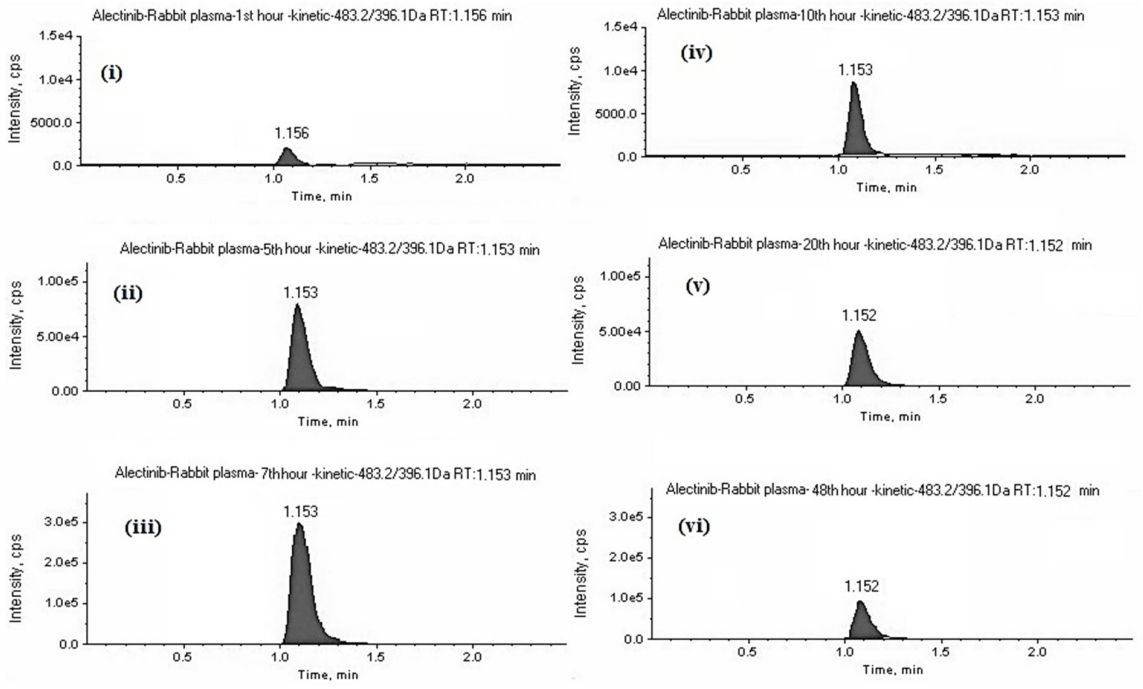

Fig. 4. MRM Chromatograms of alectinib within rabbit plasma sample for kinetic study at 1st hour (i), 5th hour (ii), 7th hour (iii) 10th hour (iv), 20th hour (v) 48th hour (vi) blood sample .

Both the alectinib and internal standard MRM chromatograms that were obtained had good peak shapes and were extremely sensitive. According to the US-FDA bio analytical technique development guidelines, the results of every validation parameter meet the requirements for acceptance. The LLOQ and ULOQ samples of the analyte and internal standard showed 0% carryover in the carry over test, indicating that they met the acceptance criteria. Since there is no interference, the alectinib created method is selective, according to the results of the matrix and analyte selectivity analysis. The matrix impact investigation's "IS normalised matrix factors" value is satisfactory, indicating that there was no discernible matrix ion effect for alectinib and internal standard. The percent CV values and percent nominal values were both within acceptable limits, according to the results of an intra- and interday precision and accuracy analysis at all quality control levels, demonstrating that the existing technique is extremely precise and accurate. During the dilution integrity study, the precision (percent CV) and accuracy (percent nominal) values of 2 and 4 times dilution were within acceptable bounds. The dilution integrity study's findings show that sample dilution has no effect on the analyte's quality, demonstrating the sample's integrity over dilution. The ruggedness of the method when switching columns and reagents was demonstrated by the accuracy and precision values in the ruggedness study, which exhibited satisfactory accuracy and precision values in all QC levels that were within the limitations. The linearity research findings using calibration curve samples in the range of 3-600 ng/ml exhibited a best fit relationship for alectinib with a correlation coefficient (r^2)= 0.998, proving the linearity of the established method using LC-MS/MS. The mean overall recovery findings of alectinib compared to internal standards were judged to be extremely satisfactory at the LQC, MQC-1, and HQC levels, and the % recovery variances fell within allowable tolerances. The developed method is stable, as evidenced by the computed percent of stability values in stock solution stability at room temperature (25^0C) and in the refrigerator (2-8^0C, 4 days). These values were determined to be satisfactory and within the range. In bench top, wet extract, freeze thaw, autosampler, short and long term (-70^0C, 30 days) stability tests, stability samples were compared with newly prepared samples, and concentration was back estimated from the calibration curve samples. The mean percent of nominal values in the category of stability study were discovered to be within the established limit, and the percent CV values were found to be within 10%, which strongly supported the stability of all quality control samples and the stability of the created method. The newly created and verified alectinib approach performed excellently while analyzing pharmacokinetic samples. Due to the smoothness of the plasma concentration and time, all parameters may be computed. All of the kinetic parameters were well suited by a single compartmental model. The pharmacokinetic profile of alectinib in rabbit plasma samples could be ascertained using the current approach with sufficient selectivity, sensitivity, and specificity.

3.Conclusion

The current method has a higher throughput than the previously published HPLC method. With less run time, the total analysis time is required to be much less. This method became more practical and cost effective because of a simple liquid-liquid extraction procedure. Empirical evidence of all validation results showed that the method is highly validated and

simple, as all parameters are within the US-FDA guidelines' acceptance limits. Overall, the complexity, sample loss, and co-precipitation issues of the earlier described methods were resolved. The sensitivity has enhanced since the lower limit of quantitation has been lowered and in the present procedure, an easily accessible and appropriate internal standard has been adopted. As a result, this current attractive, simple, and reliable novel method is unquestionably highly applicable for palbociclib quantitative analysis during clinical trials, preclinical trials, forensic, and toxicological studies.

4. Experimental

Hetero pharmaceuticals limited, Hyderabad, India, kindly gave alectinib (99.89% pure) and Palbociclib standard (99.95% pure) as a gift sample. We bought phosphate buffer from Sigma-Aldrich in Hyderabad, India. Water of the HPLC grade was purchased from Millipore Mili-Q.

High Performance Liquid chromatographic operating conditions

An isocratic elution technique was adopted with the mobile phase (Acetonitrile: water (0.1% formic acid) in the volume ratio of 75:25) derived at a flow rate of 600 µl/minute using waters Symmetry shield RP C-18 column with 4.6 mm internal diameter, 5 µm particle size, $100A^0$ pore size.

4.1. Mass spectrometry operating conditions

Mass spectrometry was performed using an API-3000 triple quadrupole mass spectrometer (AB SCIEX, Foster city, CA/concord, Ontario, Canada) was equipped with an electrospray ionization source (ESI), operating in the positive ion mode at 700^0C dessolvation temperature. The ion source voltage was 5000 V, the source temperature was maintained 380^0C. The entrance potential and collision energy was maintained 10 V and 35 V. All other tuning parameters were set for the Alectinib and internal standard palbociclib. Detection of the ions were carried out in multiple reaction monitoring by monitoring the transition pairs of m/z and m/z +447.5 (parent) →380.1 (product) for palbociclib as internal standard.

4.2. Preparation of calibration curve and quality control sample

The calibration curve samples and quality control samples of alectinib were prepared from the standard aqueous stock solution of 1 mg/mL. Further dilution was made and finally plasma spiked calibration curve samples were prepared in the range of 0.5 to 600 ng/mL. Quality control samples of alectinib were prepared by diluting standard stock solution (1 mg/mL) in the range of 1000 to 5 ng/mL of aqueous solutions. Then plasma spiked quality control samples were prepared by the mixing a definite volume of rabbit plasma to the diluted solutions to obtained the concentrations in the range of 500 ng/mL (DIQC), 300 ng/mL(HQC), 200 ng/mL(MQC-II), 100 ng/mL(MQC-I), 10 ng/mL(LQC), 0.5 ng/mL(LLOQ) level.

4.3.Preparation of mobile phase

For the preparation of 100 mL of mobile phase, 75 mL of HPLC grade acetonitrile was transferred in to clean and dry volumetric flask. 25 mL of ultrapure water (0.1% formic acid) was slowly added to the flask to achieve the final solution of mobile phase (acetonitrile: ultrapure water, 75:25).

4.4. Sample preparation

A simple liquid-liquid extraction (LLE) method was developed for sample processing. For the calibration curve and quality control samples, the alectinib plasma samples and the internal standard (palbociclib) were thawed at room temperature. Vortexed the samples to ensure full blending. With the exception of blank plasma samples, where only 25 µl of diluent (mobile phase) was added, 250 µl of alectinib plasma sample and palbociclib internal standard were pipetted out and placed in 15 ml of stoppard flask. 5 ml of tetra butyl methyl ether was used as an extraction solvent, and each tube was shaken at 400 rpm for 25 minutes using a reciprocating shaker. The samples were further centrifuged at 4000 rpm for 10 minutes at room temperature. The organic supernatant layer was transferred to pre-labeled tubes and evaporated to dryness at 40^0C using nitrogen. Both the prepared samples were reconstituted with 100 µl of mobile phase, transferred to autosampler loading vials, and 10 µl of the sample was injected into the LC-MS/MS device.

4.5.Method validation

4.5.1. Carry over effect

The autosampler was used to monitor the carryover effect of alectinib and the internal standard (palbociclib). Six replicates of analytes and internal reference samples were analyzed at the null, ULOQ, and LLOQ levels. The processed samples were inserted in a particular order, for example, LLOQ samples first, then ULOQ, then blank. At the retention of analytes and IS, the interfering peak response of blank samples should not exceed 5% of the average IS response.

4.5.2. Matrix effect

This study was performed at the LQC and HQC levels. For this study, one haemolytic and one lipemic plasma lot were chosen. One set of each sample was spiked with blank matrices (haemolytic), while the other set (LQC and HQC) with internal standard was spiked with lipemic plasma. Six replicates of aqueous samples with final LQC and HQC concentrations were prepared by adding an internal standard to reconstituted alectinib and injecting each one separately. By dividing the peak response area in the presence of matrix ion with the mean peak area response ratio in the absence of matrix ion, an internal standard normalised matrix factor was determined. The IS-normalized factor's variability, as determined by the coefficient of variance, should be less than 15%.

4.5.3. Precision and accuracy

Using several alectinib QC samples at the level of LLOQ, LQC, MQC(I and II), and HQC in six replicates, intra and interday precision and accuracy were determined, and the concentrations in these levels were measured, followed by standard deviation, percent CV for the precision, and percentage nominal for accuracy for the each replicate of alectinib sample. The acceptance requirements for accuracy (percent nominal) are 15% and 20% (for LLOQ level), respectively, and precision (percent CV) should be within 15% and 20% (LLOQ sample, respectively).

4.5.4. Linearity

The linearity of the current approach was also tested in the alectinib concentration range of 5 to 1000 ng/ml. The CC (calibration curve) samples were prepared and processed by spiking rabbit plasma. To produce the best fit for the concentration/response relationship, a regression equation with a weighing factor $1/(\text{concentration ratio})^2$ of the drug to internal normal concentration was used to create the calibration curve. The r^2 (coefficient of correlation) should be less than 0.99 as an acceptance criterion for linearity.

4.5.5. Matrix selectivity and specificity

Matrix selectivity study was tested for alectinib by analyzing plasma from six separate lots to see if the analytes' and internal standard's retention times interfered. The intervention at the drug retention times was assessed by comparing the response in blank plasma to the response of LLOQ. The intervention at the internal standard's retention time was also compared to the response of the extracted internal standard in the LLOQ study. The interfering substance's response will be considered appropriate if it is less than 20% of the mean drug response in the LLOQ sample and less than 5% in the internal standard.

4.5.6 . Recovery study

Three concentration levels LQC, MQC-1, and HQC were used in this study. Both extracted and non-extracted specimens have been packaged. At each level, internal criteria were used, and samples were processed and administered. Internal criteria were used at every stage of the production of the LQC, MQC-1, and HQC samples for the extraction of samples. Samples were processed and injected, and the percent of recovery was calculated separately for the analytes and internal standard. On average, the overall recovery was calculated.

4.5.7. Dilution integrity study

For the dilution integrity study of the alectinib was processed and 12 sets of DIQC samples of alectinib sample was prepared at 500 ng/ml. Six sets of samples were diluted twice, and another six sets of samples were diluted four times. These alectinib in samples were analysed and concentrations were calculated by multiplying the concentration by 2 in the case of a 2 times dilution and multiplying by 4 in the case of a 4 time dilution.

4.5.8. Ruggedness study

One accuracy and accuracy batch of samples was created and carried out in order to evaluate the robustness of the established LC-MS/MS method of alectinib. After being processed, these were injected into the LC-MS/MS apparatus. Different batch reagents (acetonitrile, ammonium format, and HPLC grade water) were used to produce the sample and mobile phase, as well as the same type and brand of other column on several instruments.

4.5.9. Stability studies

Other stability tests of alectinib (Bench top, wet extract, freeze thaw, autosampler, short term and long term stability) were carried out using freshly manufactured calibration curve samples and quality control samples at medium, intermediate, and high levels. These samples were then analyzed. Utilizing information from concentration response linearity, the

concentration of stability samples was estimated. The concentration of stability sample was determined using data on concentration response linearity.

4.5.10. Room temperature stability study

Alectinib stock solution that had been prepared for at least 6 hours was used for the experiment. Internal standard and analyzer stock solutions were made from scratch. The ultimate concentrations of the stock solutions (the stability samples) and fresh stock solutions (the comparison sample) are equivalent to the internal norm and middle quality control analytes. Six replicates of fresh and comparison materials were immediately injected, and the % of stability was then calculated.

4.5.11. Refrigerator stock solution stability

Six copies of the stock solution were made and kept in the refrigerator at 2-8^{0}C for four days to test the stability of alectinib. On the day of the assessment, a brand-new reference stock solution (comparison sample) was made that was identical to the MQC concentration of the analytes in the reconstituted solution and the internal standard's final concentration. Samples for stability and contrast were injected simultaneously. The stability percentage for alectinib was calculated for the analytes and internal normal.

4.5.12. Bench top stability

From the deep freezer, six sets of LQC and HQC alectinib samples were collected and left there for 12 hours without being processed. After that, six sets of fresh calibration samples and quality control samples (low, middle, and high) were made. Along with fresh samples, samples of alectinib stability were processed and analyzed. The concentration was measured using the linearity data.

4.5.13. Autosampler stability

Six sets of alectinib quality control samples were created at the LQC and HQC levels and stored in an autosampler for three days to verify type stability. At the low, middle, and high levels, all stability samples were compared to freshly made spiked calibration curves and quality control samples.

4.5.14. Freeze thaw stability

To ascertain it, four freeze-thaw cycles were performed. Six replicates of the alectinib LQC and HQC samples were made, and they were kept in a deep freezer at -70^{0}C. After 24 hours, the first six samples were taken out and allowed to defrost at room temperature before being frozen once more. After the subsequent 12 hours, the remaining samples were taken out and refrozen for an additional 12. Each sample underwent four cycles of processing. Medium, mid, and high levels of alectinib stability samples were assessed alongside newly spiked calibration samples and quality control samples.

4.5.15. Wet extract stability

To examine the stability of alectinib wet extract, six replicates of LQC and HQC samples were made, examined, and kept at room temperature for a day. After a proper stability period, the samples were injected at medium, moderate, and high doses with freshly spiked calibration curve samples and quality control samples. The total number of analytes in stability samples was calculated and compared to freshly generated samples.

4.5.16. Short term stability at -20^{0}C

Six sets of alectinib quality control samples at low and high concentrations were made after spiking, and they were placed in a deep freezer and frozen at -200C. On the day of the evaluation, the samples were processed three days later, along with freshly made calibration curve samples and quality control samples at every stage. In comparison to freshly created samples, the concentrations of the stability samples were assessed.

4.5.17. Long term stability at -70^{0}C

For a period of 30 days, alectinib LQC and HQC samples were stored at -70^{0}C to investigate this. On the day of the evaluation, six sets of long-term quality control samples (LQC and HQC) were extracted and processed using newly made calibration curves and quality control samples. The stability samples were all quantified using the calibration curve data. At each quality control level, all stability samples must have a mean percent nominal concentration that ranges from 85 to 115 percent and precision that is less than 15% of the CV percent. The stability QC samples need to be within 15% of their nominal values in at least 67% of the cases.

5.3. Pharmacokinetic study

The in vivo experimental research was carried out in compliance with the local ethical committee on animal experimentation's guidelines. The kinetic testing of alectinib was conducted on three healthy rabbits weighing 2.93 kg, 2.33 kg, and 2.89 kg and the study protocol was approved by the animal ethical committee no Approved proposal no.: IAEC/IPT-42/PN-4/Approved/4/2022). Using the oral method of administration, 3.26 mg/kg of alectinib was administered. The dose for the rabbit was measured in accordance with US FDA recommendations[15]. In order to provide the measured dose, an equivalent volume of tablet powder was converted into a suspension. An intubation tube made of silicone rubber was used to administer the suspension to each group. At 0, 1, 2, 5, 7, 10, 20, 40, and 48 hours, blood was taken from the ear vein into polypropylene K3 EDTA coated tubes as an anti-coagulant. After centrifugation, plasma was collected and maintained at -20^{0}C. The method development team's sample preparation process was used to remove the drug alectinib from the plasma samples and put it into an autosampler tube for additional analysis. The medication concentration was evaluated after each hour of blood collection. With the help of the application PK Solver 2, you can solve issues rapidly (a menu-driven adding programme for MS excel)[16]. A time vs. concentration graph was created after measuring the medicine concentrations at several points during the blood sample collection process, and pharmacokinetic features were computed.

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