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Quantitative estimation and validation of Alectinib hydrochloride drug substance using quantitative nuclear magnetic resonance spectroscopy

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Abstract

Nuclear magnetic resonance spectroscopy has been recently used for quantitative estimation of drug substance which has numerous merits in pharmaceutical applications when compared to traditional methods of chromatography. Present study focuses on method development and validation of quantitative proton nuclear magnetic spectroscopy method for estimation of alectinib hydrochloride drug substance. This method involves use of internal standard in presence of alectinib hydrochloride. For quantitation, 1H NMR signals at 8.4 ppm and 6.7 ppm corresponding to analyte proton of alectinib and ethyl 4-(dimethyl amino) benzoate internal standard respectively were used. Moreover, this method was validated as per ICH guidelines for accuracy, precision, linearity, limit of detection, limit of quantitation, range and robustness. Assay estimation by Q-NMR is facile and time-efficient which does not require reference standard of analyte and yet accurate and precise alternative as compared to estimation by other chromatographic techniques.

Keywords: Alectinib; quantitative NMR; Q-NMR; Alectinib NMR; Alectinib Quantification

1. Introduction

Alectinib hydrochloride (ALECENSA, Hoffmann-La Roche, Inc./Genentech, Inc.) received accelerated approval in December 2015 and regular approval in November 2017 by the Food and Drug Administration (FDA) for the treatment of anaplastic lymphoma kinase (ALK)-positive metastatic non-small cell lung cancer (NSCLC). Since this is recently approved anti-cancer drug, it is not official in many pharmacopoeias and therefore primary reference standard of alectinib hydrochloride is not available by them. This lack of official reference standards has driven the need for alternative analytical methods [1]. Alectinib's enhanced efficacy compared to crizotinib, especially in treatment-naive and crizotinib-resistant ALK-positive NSCLC, highlights its clinical significance and underscores the necessity for reliable analytical techniques [2]. Alectinib is a selective ALK inhibitor that targets and inhibits the ALK tyrosine kinase, preventing cancer cell proliferation [3]. Recent studies have shown that alectinib has a superior safety profile and higher potency compared to older ALK inhibitors [4]. This is the primary requirement to develop quantitative NMR method for estimation of assay of this drug which does not require its primary reference standard. The quantitative estimation by NMR was reported as early as in 1963 by Forbes and Hollis [5], [6] , since then many reports have been published involving this concept. Nonetheless, even after that the quantitative estimation was not widely accepted until two decades ago when thorough demonstration of validation of Q-NMR method was proposed by G. Maniara et. al. [7]. Soon after that, the potential of NMR as quantitative tool in organic substances was proposed to Consultative Committee for Amount of Substance (CCQM) 1998 [8], [9]. Recent developments in high-field and multi-dimensional NMR techniques have further refined qNMR methodologies, improving accuracy and sensitivity for quantitative analyses [10]. These advancements include the use of novel internal standards and improved calibration techniques that enhance the robustness of qNMR applications in pharmaceutical analysis [11]. New approaches in qNMR include the use of external calibrations and advanced data processing techniques, such as Bayesian analysis, which have further enhanced the precision of

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quantitative estimations, particularly in complex mixtures[12]. Since then, many reports were published in this realm using various nucleus such as 1H, and 31P and accepted as a tool for quantitative estimation [13], [14]. Recent developments have further refined qNMR methodologies, making them more accessible and reliable for quantitative analysis in pharmaceutical applications[15]. In 1H quantitative NMR estimation technique, the proton signals of the known weighed compound is to be compared with the proton signals of known weighed internal standard. The basic principle of the estimation by Q-NMR is that the intensity of the 1H NMR signal of the analyte is directly proportional to number of nuclei generating the response [16]. Additional benefits of qNMR include its nondestructive nature, allowing for further analysis using other techniques, as well as its high specificity, which is especially useful in complex pharmaceutical formulations [17]. A recent study demonstrated the applicability of qNMR for real-time release testing (RTRT), allowing for faster approval of pharmaceutical batches in production [18]. By applying correction factors of exact weights, molecular weights, theoretical proton values, obtained proton signal values, and potency of internal standard, the exact assay of a compound can be calculated.

As per literature search, many analytical methods are available for estimation of alectinib hydrochloride which involves lengthy chromatographic procedures and sample preparation. [19] - [22]. The recent advancements in qNMR provide a more efficient alternative to these traditional methods offering faster analysis times and reduced sample preparation requirements [23]. Moreover, qNMR has shown higher sensitivity in detecting impurities and degradation products, offering an advantage over conventional methods like HPLC [24]. Several studies have demonstrated that qNMR can be used in stability testing, ensuring the integrity of pharmaceutical compounds over time [25]. This method is also gaining traction for batch release testing, where rapid and reliable quantification is paramount [26]. Additionally, qNMR has shown promise in environmental and toxicological studies of pharmaceutical degradation products, offering a method for tracking pollutants [27]. To the best of our knowledge, no any method of quantitative NMR for estimation of assay of alectinib hydrochloride was available in literature hence this novel method was developed which comply well within the criteria of method validation as per ICH guidelines. The application of qNMR in this context aligns with recent FDA recommendations to explore alternative analytical techniques for newly approved drugs lacking reference standards. [28]. This aligns with the broader trend in analytical chemistry to seek cost-effective, accurate, and time-saving techniques in the pharmaceutical industry [29]. qNMR's increasing application for the determination of enantiomeric purity has also garnered attention, providing a non-destructive alternative to traditional chromatographic methods like HPLC and GC [30]. Furthermore, the implementation of automated qNMR systems has significantly reduced the potential for human error in the process, ensuring more consistent and reliable results in pharmaceutical applications[31]. Advanced studies have also highlighted qNMR's role in characterizing drug-drug interactions in formulations, further proving its versatility in pharmaceutical analysis [32].

2. Materials and methods

2.1. Chemicals, reagents and Materials

The active pharmaceutical ingredient alectinib hydrochloride was procured from Sun Pharmaceuticals Industries Limited., Vadodara, India. The internal reference standard ethyl 4-(dimethyl amino) benzoate was procured from Sigma-aldrich.

2.2. Instrumentation and analytical condition

2.2.1. Nuclear magnetic resonance spectrometry

The proton NMR experiments were performed on Bruker AVANCE 500 MHz NMR instrument. The probe temperature was set as 298K throughout experiment cycle. The chemical shifts of ^{1}H and ^{13}C spectra were recorded on delta scale in ppm with reference to tetra methyl silane (TMS). The axis of the scale was calibrated as 2.56 ppm for DMSO-d6 peak in ^{1}H spectra and at 39.5 ppm for DMSO-d6 peak in ^{13}C NMR spectra. 48 scans were collected for each sample with 66560 data points using a 30°_pulse length; the spectral width 10000.00 Hz; pre-acquisition delay 6 ms and acquisition time 3.3280001 s. A delay time of 10 s between pulses was used to ensure full T1 relaxation of protons. High precision NMR tubes (5 mm diameter and 7 inch length) were used for all the experiments.

2.3. Sample preparation

2.3.1. Ethyl 4-(dimethyl amino) benzoate IS standard preparation for specificity

An accurately weighed 5.0 mg of ethyl 4-(dimethyl amino) benzoate standard was transferred in to NMR tube and dissolved in 0.6 mL of DMSO-d6 diluent.

2.3.2. Alectinib hydrochloride sample preparation for specificity

An accurately weighed 10.0 mg of alectinib hydrochloride drug substance was transferred into NMR tube and dissolved in 0.6 mL of DMSO-d6 diluent.

2.3.3. Alectinib hydrochloride sample preparation in presence of ethyl 4-(dimethyl amino) benzoate IS

An accurately weighed 2.0 mg of ethyl 4-(dimethyl amino) benzoate standard IS and 3.0 mg of alectinib hydrochloride drug substance were transferred into NMR tube and dissolved in 0.6 mL of DMSO-d6 diluent.

2.3.4. Alectinib hydrochloride sample preparation in presence of ethyl 4-(dimethyl amino) benzoate IS for method validation parameters

System suitability

Although NMR instrument is very sensitive and specific, the proton signals peak shape and splitting pattern itself suggest the proper shimming and other appropriateness in NMR. However, as part of quantitative estimation, some instrumental observations could be set as system suitability parameters which must be within the limit for legitimate NMR spectra results. Such instrumental observations which could be considered as system suitability criteria are % relative standard deviation of the proton signal values obtained from replicate spectra should be not more than 2.0%, signal to noise ratio should be not more than 150, and the difference of delta ppm value of the analyte signal value should be not more than 0.2 ppm [33].

Linearity, LOD and LOQ

Triplicate sample solutions were prepared for each of 25%, 50%, 75%, 100%, 125% and 150% level of alectinib concentration.

Accuracy and Method precision

Triplicate sample solutions were prepared for each of 50%, 100% and 150% level of alectinib concentration. Sample weights were changed to achieve 50% and 150% level of alectinib concentration keeping IS weight constant so that change in proton signal value can be recorded and further calculation of changed assay can be done.

Robustness

Robustness study was carried out by challenging the different peak integration of the analyte as well as of the IS. In 1H NMR spectra, all the proton signals vary slightly in terms of producing intensity of the proton signal. This slight variation in the proton signal should not affect the quantitation of the analyte. Hence, 1H signal at 3.9 ppm was integrated instead of 8.4 ppm for the analyte and 1H signal at 4.3 ppm was integrated instead of 6.7 ppm for the IS.

2.3.5. Calculations

The amount of alectinib hydrochloride obtained and assay of the alectinib hydrochloride shall be calculated as per following equations **[33]**:

$$WtAna = \frac{IAna}{IStd}\frac{NStd}{NAna}\frac{MWAna}{MWStd}WtStd \quad \text{---------- (1)}$$

$$\%Assay = \frac{IAna}{IStd}\frac{NStd}{NAna}\frac{MWAna}{MWStd}\frac{WtStd}{WtAna}PStd \text{ ---------- (2)}$$

where WtAna= Weight of alectinib hydrochloride drug (mg), WtStd = Weight of internal standard (IS) (mg), IAna = Integral value of 1H signal due to analyte, IStd = Integral value of 1H signal due to internal Standard (IS) (2.0000), NAna = Theoretical integral value of 1H signal due to analyte (1.0000), NStd = Theoretical integral value of 1H signal due to internal standard (IS) (2.0000), MWAna = Molecular weight of analyte (519.10 g/mole), MWStd = Molecular weight of internal standard (193.24 g/mole), PStd = Potency of internal standard (IS) (99.98%)

3. Results and discussion

3.1. Spectroscopic aspects of alectinib hydrochloride and ethyl 4-(dimethyl amino) benzoate (IS) with respect to proposed Q-NMR method

Even though 1H NMR spectrum and assignment of proton signals to the various protons in the molecular structure is reported in the literature [34], Proton 1H NMR spectrum was recorded for alectinib hydrochloride in diluent of DMSO-d6 and the proton signals of 1H spectrum of alectinib hydrochloride was tried to be correlated with its structure which is in agreement with the reported results [Figure-1, Figure-2], [Table-1]. Although many internal standards available, ethyl 4-(dimethyl amino) benzoate (IS) was selected for this method due to some reasons such as, (i) it is having good solubility in DMSO-d6 solvent in which analyte is freely soluble, (ii) there are multiple 1H signal peaks available separate from analyte signal which gives multiple choice of selection of different IS and analyte peaks, (iii) this IS not reactive with analyte or with the diluent. The crucial point that needs to be addressed while optimizing the Q-NMR method is that the signals due to analyte and internal standard must not be overlapped with each other at least in the region where both the peaks are to be selected for the calculation [35]. If this separation is not achieved in the 1H NMR spectrum, the easiest way to achieve is to select alternative internal standard with similar solubility pattern so that difference in molecular structure can easily resonate 1H signals in the different isolated regions in the spectrum. For identification of 1H signals of ethyl 4-(dimethyl amino) benzoate (IS), certificate of analysis provided by Sigma-aldrich was referred which indicates that there are total 5 distinct 1H signals observed in its 1H NMR spectrum [36]. Each of these signals were tried to be correlated with its structure [Figure-3, Figure-4]. Although there are many separate 1H NMR signals of analyte and IS, signals at 8.4 ppm and 6.7 ppm corresponding to analyte proton of alectinib and ethyl 4-(dimethyl amino) benzoate internal standard respectively were used for calculation of assay [Figure-5]. As per the 1H NMR spectrum and molecular structure of alectinib hydrochloride, there are 5 aromatic protons available in the structure designated as 1, 4, 15, 16, and 18 which resonated between 7.0 ppm to 8.5 ppm. Furthermore, from the spectrum of Q-NMR it also evident that each of these proton peaks are clearly isolated from the IS peak in the nearby region. In general, protons from clear aromatic and aliphatic regions are preferred choice as it is easy to identify and integrate such peaks due to their specific splitting pattern and resonance in specific region in the spectrum. Although the resonances between 1.2 ppm to 4.5 ppm were observed to be more or less overlapped with internal standard peaks and not chosen as the candidates for calculation by Q-NMR, selection of other potential proton signals were challenged in robustness study during method validation and found satisfactory.

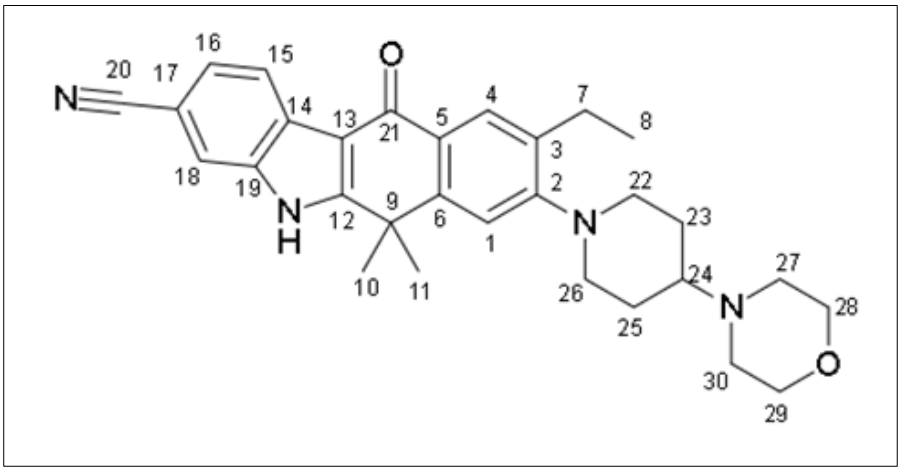

Figure 1 Molecular Structure of Alectinib

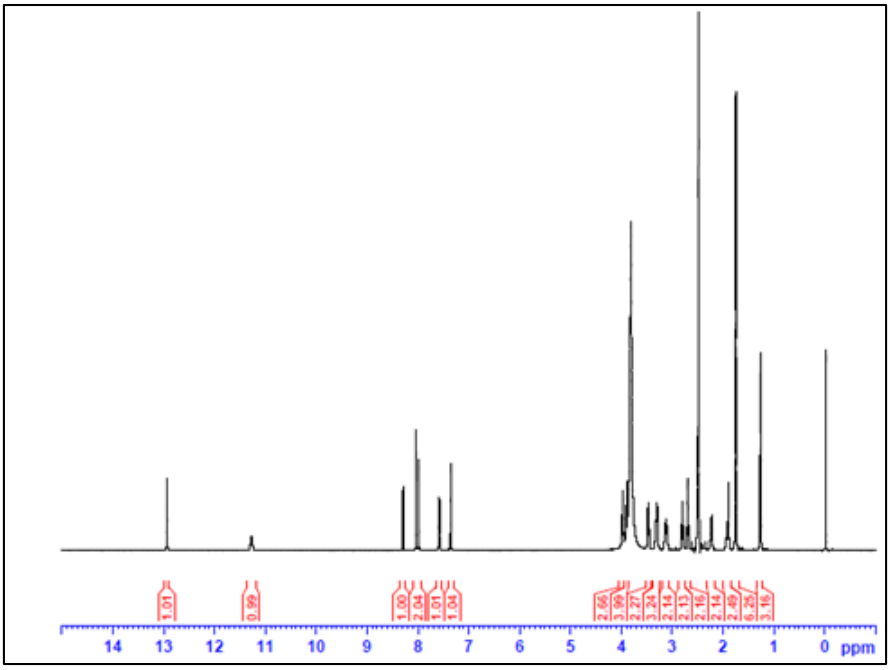

Figure 2 1H NMR Spectrum of alectinib hydrochloride

Table 1 Specroscopic aspects of 1H NMR of alectinib hydrochloride

Chemical shift (ppm)	Multiplicity	No. of protons	Assignment in structure
1.34	Triplet	3	8
1.82	Singlet	6	10, 11
1.90-4.20	Multiplet	19	7, 22 to 28, 29, 30
7.35-8.45	Multiplet	5	1, 4, 15, 16, 18
10.79	Singlet	1	Hydrochloride
12.88	Singlet	1	NH
Total no. of protons		35	-

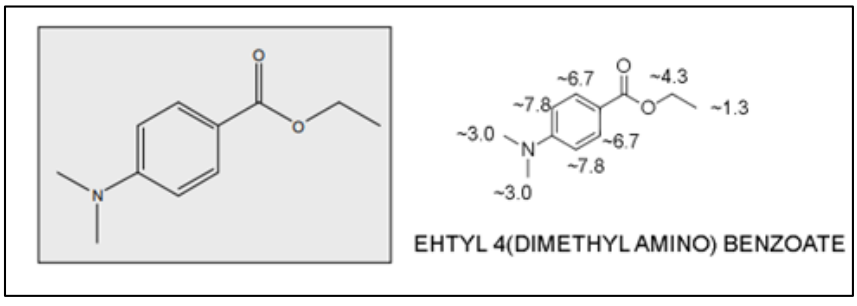

Figure 3 Molecular Structure of ethyl 4-(dimethylamino) benzoate

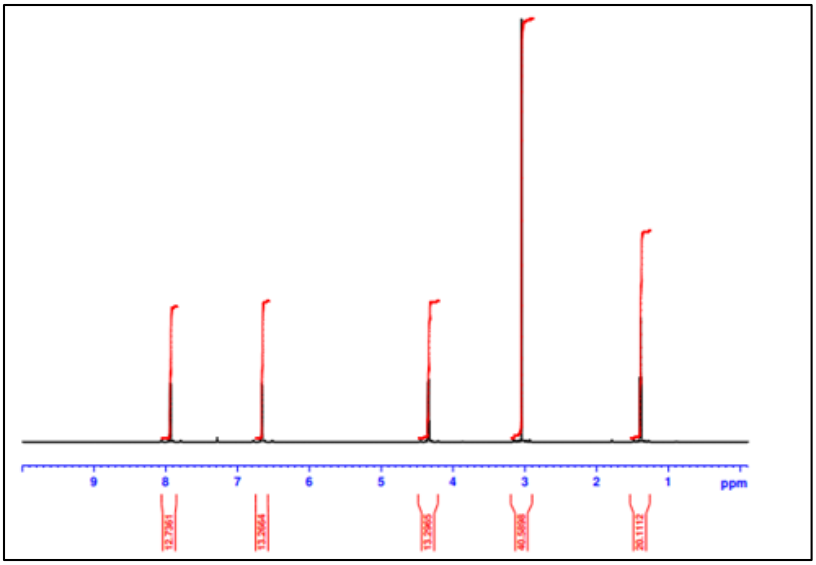

Figure 4 NMR spectrum of (600MHz,ethyl 4-(dimethyl amino) benzoate in CDCL3) from Certificate by sigma Aldrich

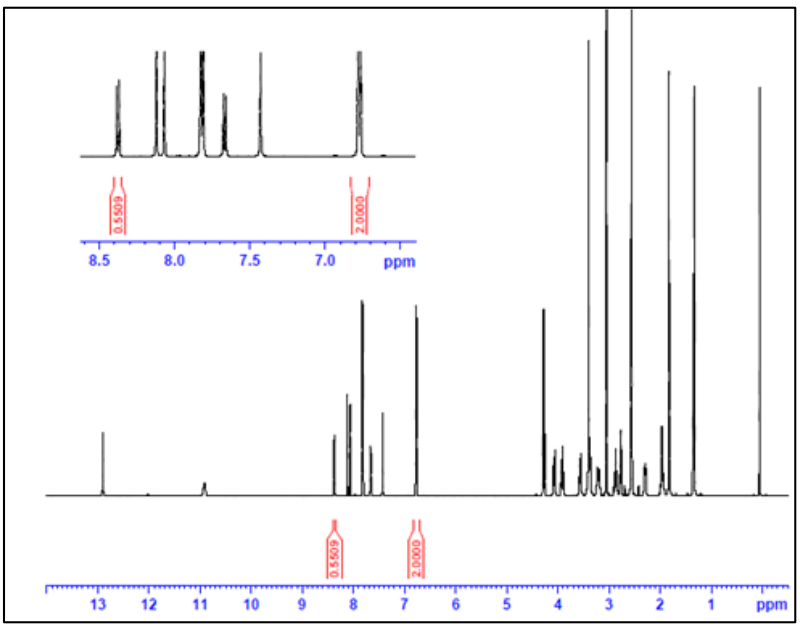

Figure 5 1H NMR spectrum of Alectinib hydrochloride inn presence of IS for Q-NMR at 100% level

3.2. Method validation

The method was validated as per International Conference on Harmonization (ICH) guidelines and following parameters were covered: system suitability, linearity, LOD, LOQ, accuracy, method precision, intermediate precision, range and robustness.

3.2.1. System suitability

System suitability criteria of % relative standard deviation of all the applicable replicates were obtained less than 2.0% throughout all the validation parameter study. Moreover, the delta ppm value of the target signals of analyte as well as IS were also recorded and did not deviate more than 0.2 ppm.

3.2.2. Linearity

Linearity was performed by analyzing sample solutions containing alectinib concentration from 25% to 150% range each in triplicate. Linearity curve was plotted between weight of alectinib taken and the integral value of 1H NMR signal at 8.4 ppm from the level of 25% to 150%. The equation of the curve was obtained as y = 0.18220x – 0.00962 and the correlation coefficient was found to be 0.9997 indicating good linear response of the analyte proton signal, [Table-2] [Figure-6].

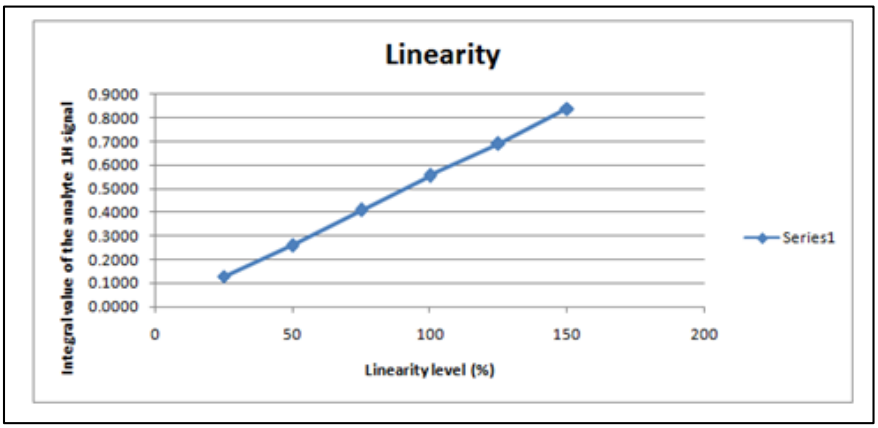

Figure 6 Caliberation curve for Linearity

Table 2 Linearity results

Linearity level	Sample weight (mg)	Integral value of the analyte 1H signal
25%	0.7550	0.1275
25%	0.7561	0.1294
25%	0.7521	0.1280
50%	1.5070	0.2598
50%	1.4810	0.2568
50%	1.5125	0.2611
75%	2.2440	0.4076
75%	2.2612	0.4115
75%	2.2589	0.4101
100%	3.0990	0.5509
100%	3.1250	0.5590
100%	3.1514	0.5569
125%	3.8600	0.6888
125%	3.8972	0.6920
125%	3.8200	0.6902
150%	4.7030	0.8389
150%	4.6159	0.8414
150%	4.5985	0.8348
	Standard deviation	0.2506
	Slope (S)	0.18220

	Intercept	-0.00962
	Correlation Coefficient (r)	0.9997

3.2.3. LOD and LOQ

LOD and LOQ were determined by the value of standard deviation of the response (σ) and slope (S) from the linearity curve plotted from 25% to 150% level. The standard deviation (σ) and slope (S) was obtained to be 0.250568 and 0.18220 respectively, hence LOD and LOQ were calculated as per equation (1) and (2) and obtained as 4.54% and 13.75% level respectively.

$$LOD = \frac{3.3 * \sigma}{S} \text{ ---------- (3)}$$

$$LOQ = \frac{10 * \sigma}{S} \text{ ---------- (4)}$$

3.2.4. Accuracy and Method precision

As per ICH guidelines, accuracy and precision was established by analyzing nine different sample preparation of triplicate sample preparation at three different concentrations of 50%, 100% and 150%. Amount of drug added and amount of drug obtained was calculated and thereby %assay was calculated which were found to be well within the range between 96.54 to 97.68% and %RSD was also found to be 0.40% indicating the analytical method found to be accurate and precise. [Table-3].

Table 3 Accuracy and Method precision test result

Accuracy level	**Amount of drug added (mg)**	**Amount of drug obtained (mg)**	**%Assay**
50% Set-1	1.51	1.46	96.54
50% Set-2	1.48	1.43	96.79
50% Set-3	1.51	1.47	96.97
100% Set-1	3.10	3.02	97.35
100% Set-2	3.13	3.04	97.29
100% Set-3	3.15	3.07	97.35
150% Set-1	4.70	4.59	97.54
150% Set-2	4.62	4.51	97.68
150% Set-3	4.60	4.49	97.59
Mean			97.23
Standard deviation			0.39
%RSD			0.40

3.2.5. Range

From the results obtained from linearity, LOD, LOQ, accuracy, method precision, the range of the analytical method was obtained from 13.75% to 150% level concentration of alectinib hydrochloride.

3.2.6. Robustness

Even after selecting different 1H signal peak for the analyte as well as IS, the %assay was obtained 97.42% which is almost comparable to 97.33% as per initial analysis which indicates the different peak integration of the 1H peaks do not have major impact on quantitation of the alectinib hydrochloride.

Abbreviations

- ICH: International conference on harmonization,
- NSCLC: Non-small-cell lung cancer,
- ALK: Anaplastic lymphoma kinase, DMSO: Dimethyl sulphoxide

4. Conclusion

The analytical method for estimation of alectinib hydrochloride by quantitative NMR satisfied all the validation requirements as per ICH guideline such as linearity, method precision, accuracy, and robustness which proves that this method would be a good alternative to previously reported analytical methods of chromatography and other spectroscopy. Apart from this, quantitative estimation by Q-NMR is very rapid, facile in sample preparation and also does not involve costly and difficult to available primary reference standard of the analyte. These merits of this method make it really useful for the pharmaceutical industry to utilize this method in quality control and other pharmaceutical applications.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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