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Original Article

LC-MS/MS and NMR Studies for Identification and Characterization of Forced Degradation Products of Acalabrutinib

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ABSTRACT

A new method named "Stability Indicating LC-MS/MS" was developed to make analysis on acalabrutinib and this method was stable for this anticancer drug. In acid, basic and oxidation the drug was shown instability on these stress conditions. Acalabrutinib was used to form four degradation products and the developed method was used to separate based on "Zorbax C_{18} column (150 mm x 4.6 mm, 5 µm)" and for this separation the technique isocratic elution was used with the flow rate of 1.0 mL/min. It was found that the new developed method was valid as per the guidelines of ICH. A pathway known as fragmentation of drug was founded initially to identify the formed degradation products and in this identification LC-MS/MS method was used and also fragmentation studies were used. After the identification, isolation of the degradation products was done and (^{1}H-NMR) was subjective for this process. The environment of basic, acid and oxidative were used to obtain the degradation products and these were isolated by the advanced techniques known as acid degradation product (DP1) of acalabrutinib with a molecular mass of 390.200 Da, empirical formula $C_{21}H_{19}N_5O_3$ with name as (S)-4-(8-amino-3-(1-(but-2-ynoyl) pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl) benzoic acid". 442.100 Da is the molecular mass of base degradation product (DP2), $C_{24}H_{23}N_7O_2$ is the empirical formula with name as "(S)-4-(3-(1-ace tylpyrrolidin-2-yl)-8-aminoimidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2-yl)benzamide". 498.400 Da is the molecular mass of another acalabrutinib base degradation product (DP3), $C_{27}H_{27}N_7O_3$ is the empirical formula which is known as "(S)-4-(8-amino-3-(1-(3-methoxybut-2-enoyl) pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2-yl)benzamide". 482.200 Da is the molecular mass of oxidation degradation product (DP4), $C_{26}H_{23}N_7O_3$ is the empirical formula which is known as "2-(4-(8-amino-3-(1-(but-2-ynoyl)pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl) benzamido) pyridine-1-oxide".

Keywords: Acalabrutinib, Isolation, Characterization, Forced Degradation Studies, LC-MS/MS, NMR.

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INTRODUCTION

Hydrolytic, photolytic, oxidation and thermal, these stress situations are considered at the time of testing stability of the substances of the drug and this is approached as an important element in the process of drug development.[1,2] ICH and many others provide guidelines for testing of stability to define the characteristics of the degradation products.[3,4] However, during the storage DPs were obtained at very low levels. As a result, different stress conditions were applied to generate the DPs in higher amounts.[5] Identification of DPs is much more critical as the total amount of this product in the mixture is low level. NMR and LC-MS/MS technique are used extensively.[6,7] Figure 1 is (4-[8-amino3-[(2S)-1-but-2-ynoylpyrrolidin-2 -yl]imidazo[1,5-a]pyrazin1-yl]-N-(2-pyridyl)benzamide)] is the chemical name of the acalabrutinib. The molecular formula of acalabrutinib is $C_{26}H_{23}N_7O_2$ and 465.5 g/mol is the molecular mass of it.[8] Acalabrutinib, is a solid dosage form, insoluble in water and soluble in ethanol, DMSO, and DMF. It is claimed that acalabrutinib can be used as pharmacological agent in novel cancer as it has high level affinity and has also inhibitor potency in Bruton's Tyrosine Kinase (BTK) proposal for using it is shown for the (MCL) patients.[9,10] In the present time it is recommended to use acalabrutinib for the adult MCL patients who have taken one therapy of it.

Acalabrutinib (Figure 1), is considered as a high level selective inhibitor of Bruton's tyrosine kinase and response rate of it high that is used for the treatment of CLL.[11] Cys481 is bound covalently by acalabrutinib in the pocket of ATP of Bruton's tyrosine kinase.[12]

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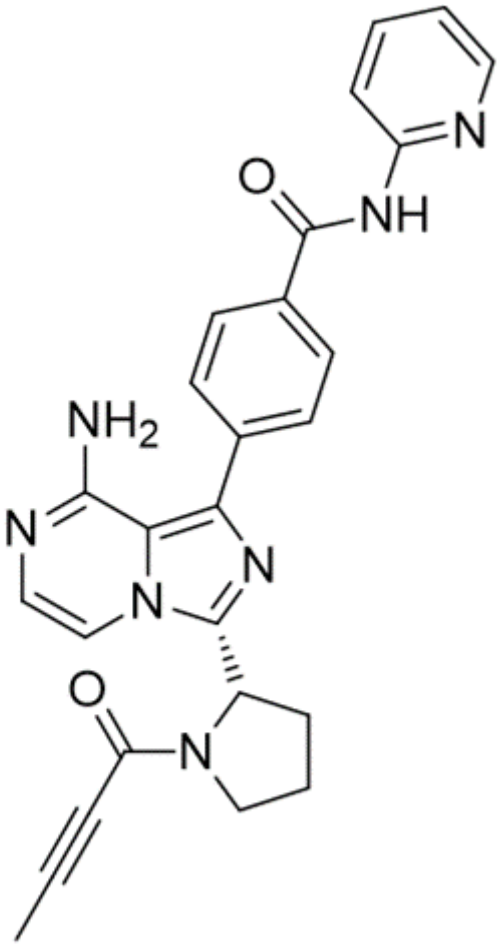

Figure 1: Structure of acalabrutinib.

Literature surveys revealed that few methods named as RP-HPLC have been reported to estimate acalabrutinib bulkily and dosages of pharmaceutical form.[13,14] Literature reviews also revealed that few bioanalytical methods are available to qualify acalabrutinib and its metabolic effect in human or plasma of animal.[15-17] But the presences of method named stability indicating LC-MS/MS was not revealed by the literature review for the acalabrutinib in bulk and dosages of pharmaceutical and for this purpose, LC-MS/MS technique was used and in this process pathways of degradation and degradation products (DPs) of acalabrutinib were reported. Hence, the findings of our current research was to: (i) ICH definition based conditions related stress studies must be carried out; (ii) validation and development of stability of the method, LC-MS/MS; (iii) fragmentation pathway of all kind of degradation products (DPs) of acalabrutinib is established and characterized; (iv) it is necessary to identify and isolate the variant of degradation products of acalabrutinib.

MATERIALS AND METHODS

Reagents and chemical

Acalabrutinib was procured from "BOC Sciences Laboratories (USA)". Analytical reagents, sodium hydroxide (NaOH) and formic acid (HCOOH) and was procured from "S.D Fine-Chem Ltd., (Mumbai, India)", hydrochloric acid (HCl) was procured from "Merck Specialties Pvt. Ltd., (Mumbai, India)", acetonitrile (ACN) and methanol (MeOH) were procured from "J.T. Baker Chemicals Pvt. Ltd., (Mumbai, India)" and hydrogen peroxide (H_2O_2) was procured from the company, "Qualigens Fine Chemicals Pvt. Ltd.", which is located in Mumbai, India. Ultra-Pure water was procured from "Aquarch" located in Ahmedabad, India was chosen in the whole study.

Instrumentation

For validation and development of a method for acalabrutinib, LC-MS/MS system was implemented in the mode of positive ion. An "Agilent 1100 (Waldbronn, Germany) LC system" equipped with thermostatted column compartment (G1316A), quaternary pumps (G1311A), auto injector and auto sampler (G1313) is associated with "triple quadrupole mass spectrometer (Applied Biosystems Sciex API 2000, USA)". ESI is the source of mass spectrometer. Analyst with the version of 1.6.2 was used for data collection. In the process of developing the method and validation column of "Agilent, Zorbax C_{18} (150 mm x 4.6 mm, 5µm)" was used for acalabrutinib. "Preparative Shimadzu HPLC" was the equipment of "LC-20AR pump and rheodyne injector" which is connected with the detector, SPD-20AT. "Chromanik C_{18} (150mm x 20 mm, 10µm)" was the column used to isolate study DPs of acalabrutinib. A 500MHz Bruker NMR based spectroscopy were implemented for making a record of the NMR spectrum of degradation products (DPs) of acalabrutinib. Other instruments such as pH meter ("Analab Scientific Pvt. Ltd.,"), an instrument for weighing balance from ("Shimadzu, ATX-224, Kyoto, Japan") oven for hot air and a chamber for photostability from ("Kesar Control System, Ahmedabad, India") were used.

Chromatographic Method

In the temperature of 35°C with the column "Agilent, Zorbax C_{18} (150mm X 4.6mm, 5µm)" chromatographic conditions of LC-MS/MS were performed. The mobile phase consist of HPLC grade water containing 0.1% of formic acid and methanol in the ratio of 50:50 v/v was used. 1.0 mL/min is the flow rate and 20µL was the injection volume in this process. The diluent used for solution preparation was HPLC grade water and methanol in the ratio of 50:50v/v. Positive mode ESI recorded mass spectra. The use of nitrogen was as curtain gas. For optimizing maximum sensitivity, IS, CUR, DP, FP, EP, GS1 and GS2 these mass spectrometers parameters were used to characterized degradation products of acalabrutinib. The column Chromanik C_{18} whose measurements was 150mm x 20mm, 10µm was used in the process of isolation with HPLC grade water in which it contains 0.1% formic acid: methanol as mobile phase with 10 mL/min flow rate at 35°C. Monitoring of the chromatogram was at 230 nm with injection volume 1000µL. The purified DPs of acalabrutinib were elucidated by ^{1}H-NMR.

Standard and stock solution preparation

Preparation of standard stock solution of acalabrutinib

Measurements of methanol was 60 mL that was mixed with 10 mg of acalabrutinib and sonicated in the flask whose volume was 100mL for making up total 100 mL mixture with methanol.

Preparation of solution known as working standard of acalabrutinib

1.0 mL standard stock solution was taken of acalabrutinib in the flask whose volume was 100 mL which was diluted with required mark with diluent.

Acid degradation solution preparation of acalabrutinib

Stock quality 1.0 mL solution was taken in a flask that is 100 mL and 1.0mL of 0.1M HCl was added with it. In the temperature of 60°C the flask was refluxed for 8 hours, cooled, neutralized with an amount of 0.1M NaOH, and the mixture was make up with the diluent.

Base degradation mixture preparation of acalabrutinib

Measurement of 1.0 mL mixture that is standard stock of acalabrutinib in the flask whose volume is 100 mL and added 1.0 mL liquid of 0.1M NaOH. The mixture was refluxed in the temperature of 60°C for 12 hours, cooled, the entire mixture was neutralized with an amount of 0.1 M HCl and make up the volume with diluent.

Oxidative degradation mixture preparation of acalabrutinib

Take 1.0 mL of standard level stock mixture of acalabrutinib in a 100 mL flask and mixed 1.0 mL of 3% H_2O_2. The solution was refluxed at 60°C for 4 hours, cooling it into normal temperature and make up the volume with diluent.

Neutral degradation solution preparation of acalabrutinib

1.0 mL of standard quality of stock mixture was taken of acalabrutinib in a 100 mL flask and mixed the amount of 1.0 mL of H_2O. The solution was refluxed at 60°C and this process took time of 72 hours, cooling down the mixture in normal temperature and make up the volume with diluent.

Thermal degradation solution preparation of acalabrutinib

Weighed about 100.0mg of acalabrutinib in petridish and placed the petri dish in hot air oven at 100°C for 5 days. Later, in the flask of 100 mL, 10 mg above sample was added 60mL methanol and sonicated to dissolve, making up mixture with methanol. Finally, the solution is prepared by transferring the solution of 1.0mL in 100mL flask and make up the volume with diluent.

Photo degradation solution preparation of acalabrutinib

100.0 mg of acalabrutinib in petri dish was placed in photo stability chamber for 5 days. Rest of the particular procedure is similar to thermal degradation solution.

Preparing sample mixture of acalabrutinib (calquence capsule)

Weight of NLT 10 capsules was considered in this process, and net content of individual capsule was calculated. Mixed the mixture contents of the capsules and a 10 mg of acalabrutinib was added to a 100 mL flask. 60 mL amount of methanol was mixed and the mixture was sonicated for the time period of 10 min, methanol was diluted with it. Finally, the solution as sample is prepared by transferring 1.0mL amount of stock mixture in 100mL flask and making up with diluent. The sample was filtered with 0.45µ PTFE syringe filter.

Forced degradation studies in sample (calquence capsule)

The sample was also performed for forced degradation studies by preparing equivalent concentration to standard preparation with same forced degradation conditions as mentioned above.

Validation

According to ICH recommendations Q2(R1), the LC-MS/MS method that was developed and verified to ensure that it satisfies the requirements for specificity, linearity, precision (repeatability, intraday precision, and interday precision), accuracy, and robustness.[18] To determine whether or not the concentration of acalabrutinib in methanol was linear, a stock solution of the compound was titrated from 0.5 to 1.5 µg/ml. There was a single injection of each solution that was 20 µL in volume. In order to calibrate the instrument, a plot of the peak area vs the matching concentrations was carried out. With the help of the calibration graph, we were able to estimate the LOD and LOQ. In order to establish the intraday precision and the interday precision, three separate analyses were performed on each concentration of drug solution (0.5, 1.0, and 1.5 µg/ml) on the same day and three days apart. In order to establish accuracy, a sample that contained a known concentration of drug was spiked with three more replicates, and the percentage of recovery was calculated. We checked the consistency by modifying the chromatographic parameters, such as the flow rate, the temperature of the column oven and the mobile phase ratio. Calculating the purity of the acalabrutinib peak allowed us to establish the level of specificity possessed by the approach. It is now possible to conduct routine analysis of the marketed formulation of acalabrutinib by using

the developed and validated LC-MS/MS method in quality control laboratories.

RESULTS AND DISCUSSION

Optimization of LC-MS/MS chromatographic condition for analysis of acalabrutinib

Researchers used forced degradation experiments to identify the degrading compounds and learn more about the stability-indicating character of the chromatographic approach. The primary goals of this study were to (1) identify and characterize forced degradation products (DPs) of acalabrutinib, and (2) develop a novel stability-indicating LC-MS/MS analytical technique for the analysis of this medication. During the optimization process, several distinct mobile phase experiments were conducted on an Agilent, Zorbax, C_{18} (150mm X 4.6mm, 5µm) column. The final mobile phase will be a 50:50 (v/v) combination of water containing 0.1% formic acid and methanol to improve the separation of acalabrutinib from its DPs. With an injection volume of 20 microliters and a flow rate of 1 mL per minute, isocratic elution was carried out at a temperature of 35°C. The time limit for the competition was 10 min. At a mass-to-charge ratio of 466.300 Da to 136.100 Da, acalabrutinib was detected positively. The curtain gas was created using nitrogen. For the ion source, we set the parameters as follows: temperature 400°C; 20 psi for the curtain gas; 5000 volts for the ion spray and 60 psi for both ion source gases 1 and 2. Prior to the start of the scan, we subjected it to these conditions: A multi-resolution (MRM) scan set to 32 volts for declustering, 400 volts for focusing, and 10 volts for entry. This enhanced LC-MS/MS method was used to successfully separate acalabrutinib from its DPs. The procedure was validated using the criteria established by ICH standards Q2 (R1). The retention time of acalabrutinib is shown to be 3.85 min, with a peak in the standard chromatogram (Figure 2).

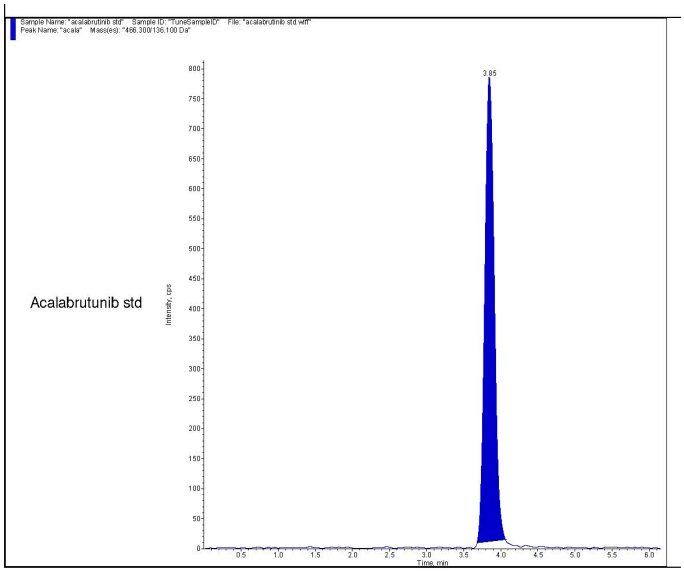

Figure 2: Standard LC-MS/MS chromatogram of acalabrutinib.

Validation

To achieve this level of accuracy, the word "acalabrutinib" is emphasized in a number of various ways. "Specificity" refers to an analytical method's capacity to deliver reliable results despite the presence of contaminants that are known to be present in the substance. Every degradant products (DPs) could be distinguished from acalabrutinib, and the approach was proven to be highly specific. The concentrations of acalabrutinib, which were measured from 0.5 to 1.5 µg/ml, were found to increase in a linear fashion. The best-fitting equation for the data was found to be Y = 80.48 x -19.12, and the calculated correlation coefficient (R^2) was 0.996 when the data was analyzed using a linear regression model. These findings demonstrate a linearity that bodes well. The analyte concentration is reflected by the LOD and LOQ values. There was a 0.142 µg/ml detection limit and a 0.430 µg/ml quantification limit. Table 1 contains data on the consistency of intraday and interday transactions. The approach's dependability is demonstrated by its intraday precision ranging from 0.655 to 1.327% and its interday precision ranging from 0.733 to 1.666%. Our accuracy was tested by introducing a sample with a known concentration of drug into it, and the results are shown in Table 2. The percentages of recovery were as high as 100.54% and as low as 99.89%. The newly created method was subjected to a series of controlled trials in which the mobile phase, column oven temperature and flow rate were all tweaked slightly to ascertain the method's reliability. The robustness test shows that the %RSD for area is less than or equal to 2.0% across all levels. The amount of acalabrutinib in the calquence capsule formulation currently on the market was calculated using the tried-and-true LC-MS/MS technique. To everyone's satisfaction, it was determined that the average percentages obtained were in reasonable agreement with the claims indicated on the packaging.

Degradation behavior of acalabrutinib on LC-MS/MS

The drug acalabrutinib was tested in a variety of environments, including those that were acidic, basic, neutral, oxidative, photo and thermal. Using LC-MS/MS, the characteristics of four different degradation products (DPs) were determined. Acalabrutinib was able to be broken down when it was exposed to acid, base, and oxidative stress. Only one degradation product, designated DP1, is produced whenever acid is used to break down any substance. Two degradation products, denoted by the acronyms DP2 and DP3, were produced as a direct consequence of the base breaking down. When it came to oxidative deterioration, there was never more than one product of degradation at any given time (DP4). The pharmacodynamics properties of acalabrutinib are unaffected by exposure to either neutral, light or temperature stress. Using LC-MS/MS, Figure 3 depicts the chromatogram of the acid, base, and oxidation degradation solution of acalabrutinib. This solution was used in the study. The retention times and m/z values of acalabrutinib degradants products (DPs) and fragment ions are listed in Table 3, as may be seen by reading that table.

Table 1: Results of Precision data of Acalabrutinib.

Conc (µg/mL)	Intraday Precision Mean Area ± SD, %R.S.D	Interday Precision Mean Area ± SD, %R.S.D
0.5	3190.908 ± 42.355, 1.327	3342.327 ± 55.687, 1.666
1.0	6280.739 ± 56.312, 0.898	6397.671 ± 67.802, 1.060
1.5	9713.271 ± 63.579, 0.655	9731.256 ± 71.347, 0.733

Table 2: Results of Accuracy Data of Acalabrutinib.

Spiked Conc (µg/mL)	Sample amount (µg/mL)	Amount Added (µg/mL)	% Recovery
80%	0.5	0.4	100.41
100%	0.5	0.5	100.54
120%	0.5	0.6	99.89

Degradation behavior of acalabrutinib on HPLC

Using the approved LC-MS/MS technique in the HPLC system led to the greatest acalabrutinib degradation and hence made degradation products (DPs) isolation easier. The chromatographic separation was carried out using an Agilent Zorbax C_{18} (150mm x 4.6mm, 5µm) column and was carried out at a temperature of 35°C with a flow rate of 1.0 mL/min and an injection volume of 20 microliters. In the used mobile phase, methanol and water were mixed at a volume-to-volume (v/v) ratio of 50:50, and formic acid was diluted to a concentration of 0.1% in the water. The detection was made at a wavelength of 230 nm. Acalabrutinib deteriorates in highly acidic, highly basic, or highly oxidised conditions. Studies showed that after 12 hours and 8 hours of exposure to acidic and oxidative stress at 60°C, acalabrutinib was transformed into less effective forms. Deterioration may also be detected after only 24 hours in temperatures of 60°C under basic stress. Acalabrutinib is stable against neutral, thermal, and photo breakdown. The chromatograms for acid, base, and oxidation degradation of acalabrutinib in the degradation solution are shown superimposed in Figure 4.

Preparation, isolation and purification of degradation products (DPs) of acalabrutinib

The 100 mg of acalabrutinib that had been weighed in was placed inside of a round-bottomed flask with a capacity of 100 mL. Following the addition of 4 mL of methanol and the use of sonication to dissolve the medicine, 1 mL of 1.0M HCl was added to the mixture. After being heated for a total of 12 hours at a temperature of 60°C, the mixture was then cooled to room temperature. The solution generated the acid degradant product (DP1) of acalabrutinib, which is an intermediary in the process of its degradation. We utilized a sonicator and a round-bottomed flask with a capacity of 100 mL to dissolve 150 mg of acalabrutinib in the methanol. After that, we put in 4 mL of methanol and 1 milliliter of 1.0M NaOH solution. After heating the solution for 24 hours at a temperature of 60°C using a process called reflux, it was then cooled to room temperature. The final product generate base degradation products (DP2 and DP3) of acalabrutinib. After weighing out 100 mg of acalabrutinib, 4 mL of methanol was added to it, and then the mixture was sonicated to cause the acalabrutinib to dissolve before 1 mL of 30% H_2O_2 was added. After heating the mixture for 8 hours to a temperature of 60°C, the atmosphere was allowed to continue to reflux while the mixture was allowed to cool to room temperature. After this step, the solution was concentrated so that the oxidative breakdown product (DP4) of acalabrutinib could be isolated.

Under the aforementioned conditions, acalabrutinib degradation products were isolated using preparative HPLC. Using chromatography at a temperature of 35°C, with a flow rate of 10 mL per minute and an injection volume of 1000 µL, the acid, base and oxidation degradation products of acalabrutinib (DP1, DP2, DP3 and DP4) were separated. The column used was a Chromanik C_{18} that measured 150 mm by 20 mm and particle size 10 µm. The mobile phase was made up of water containing 0.1% formic acid and methanol for extracting acid, base and oxidation degradation products (DP1, DP2, DP3, DP4) of acalabrutinib. The measurement was taken at a wavelength of 230 nm when it was performed. After the acid degradation process was finished and neutralized with 1.0M NaOH, lyophilizing the solution that was collected from the outlet resulted in the production of crude solid samples of the acid degradant product (DP1) of acalabrutinib. After being collected, neutralized with 1.0M HCl the outlet solution of the base degradation was put via chloroform extraction. In order to obtain the base degradant products of acalabrutinib (DP2 and DP3), we first had to wait for the organic layer to form before collecting it and then evaporating it. A crude solid sample of the oxidative degradant product was obtained by collecting and evaporating the outlet oxidative degradation solution of acalabrutinib (DP4).

Table 3: Results of Retention time and *m/z* values of DPs and its fragment ions of acalabrutinib.

Degradation Condition	Degradation Products (DPs)	Retention Time (min)	Molecular Ion *m/z* value	Selected Fragment ion *m/z* value
Acid	DP1	1.77	390.200	136.100
Base	DP2	2.01	442.100	330.300
	DP3	4.69	498.400	399.200
Oxidation	DP4	3.02	482.200	372.100

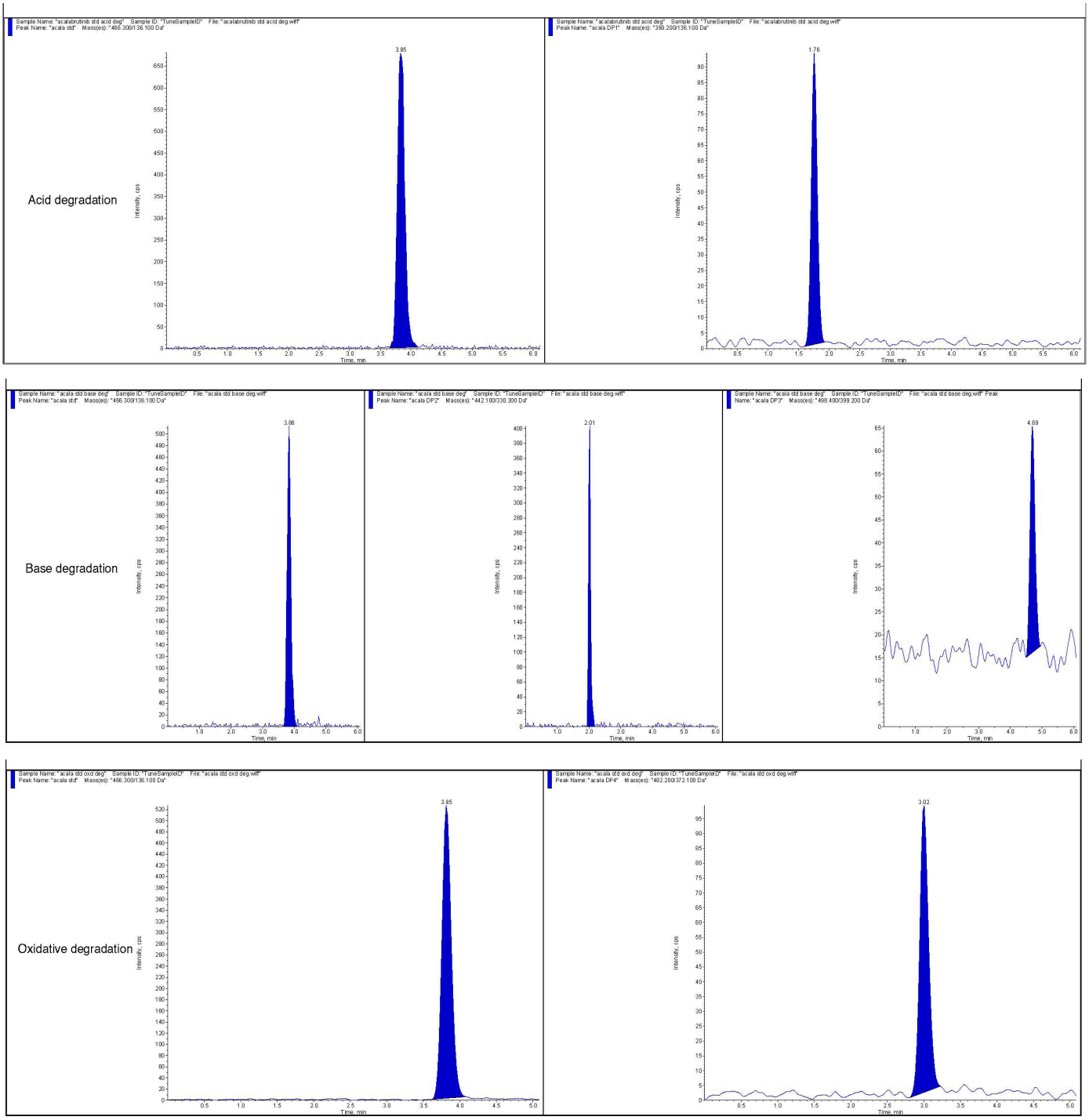

Figure 3: LC-MS/MS chromatogram of acid, base and oxidation degradation solution of acalabrutinib.

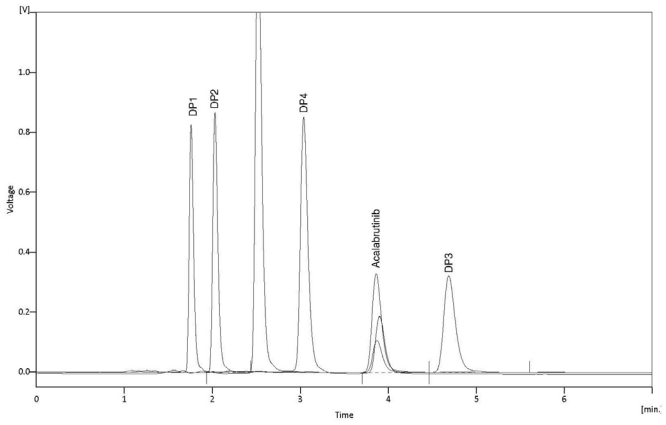

Figure 4: Overlay chromatogram of acid, base and oxidation degradation solution of acalabrutinib.

Structure confirmation of degradation product (DPs) of acalabrutinib

Unit resolution MS analysis was used to confirm the chemical structure of acalabrutinib in acidic, basic, and oxidative degradation conditions. In the mass spectral analysis of the acid degradation product (DP1) of acalabrutinib, the presence of protonated molecular ion has chemical formula $C_{21}H_{19}N_5O_3$, mass 390.200 Da and named as (S)-4-(8-amino-3-(1-(but-2-ynoyl)pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl)benzoic acid. Spectroscopic methods, including ^{1}H NMR, were used to further establish the structure of the acalabrutinib acid degradation product (DP1). The structure assigned to (DP1) is also supported by ^{1}H NMR spectra. The mass spectrum of the base degradation product (DP2) of acalabrutinib shows the presence of a protonated molecular ion with the mass number 442.100 Da, the chemical formula $C_{24}H_{23}N_7O_2$, and named as (S)-4-(3-(1-acetylpyrrolidin-2-yl)-8-aminoimidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2-yl)benzamide. Spectroscopic techniques, such as ^{1}H NMR, were used to further establish the structure of the acalabrutinib base degradation product (DP2). The proton Nuclear Magnetic Resonance (NMR) spectra of the base degradation product (DP2) of acalabrutinib matched the protons predicted by the chemical structure. The mass spectrum of the acalabrutinib base degradation product (DP3) shows the presence of a protonated molecular ion with molecular mass 498.400 Da, chemical formula $C_{27}H_{27}N_7O_3$ and named as (S)-4-(8-amino-3-(1-(3-methoxybut-2-enoyl)pyrrolidin-2-yl) imidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2-yl)benzamide. Spectroscopic techniques, such as ^{1}H NMR, were used to further establish the structure of the acalabrutinib base degradation product (DP3). Proton nuclear magnetic resonance spectroscopy showed that the structural protons of acalabrutinib matched those of its base degradation product (DP3). In the mass spectrum of acalabrutinib oxidative breakdown product (DP4), the protonated molecular ion with mass number 482.200 Da may be observed. 482.200 Da, is a chemical compound with the formula $C_{26}H_{23}N_7O_3$ and known as 2-(4-(8-amino-3-(1-(but-2-ynoyl)pyrrolidin-2-yl) imidazo[1,5-a]pyrazin-1-yl)benzamido) pyridine-1-oxide. The structure of acalabrutinib oxidative breakdown product (DP4) was confirmed using ^{1}H NMR and other spectroscopic methods. Proton Nuclear Magnetic Resonance (NMR) spectra showed that the protons in the oxidative degradation product (DP4) of acalabrutinib matched the structural protons.

Mass fragmentation pattern of acalabrutinib

During the MS analysis of acalabrutinib, two fragments were identified as being present. Prior to the MS method, a Multi-Stage (MS^n) mass fragmentation analysis was performed in order to gain a deeper understanding of the origin of each fragment. It has been calculated that the *m/z* ratio of the two protonated fragments that were discovered, one at 372.100 Da (FRAG 1) and the other at 136.100 Da (FRAG 2). The mass spectra of acalabrutinib as well as a predicted fragmentation process is shown in supplementary data as Figure S1(a) and Figure S1(b), respectively.

Mass fragmentation pattern of acid degradant product (DP1) of acalabrutinib

In acid degradation, acalabrutinib on reaction with acid undergo acid hydrolysis of the terminal amide group leading to the formation of acid degradant product (DP1) of acalabrutinib whose *m/z* ratio is obtained around 390.200 Da (DP1) and its two protonated fragmentation ion is observed whose *m/z* ratio is obtained around 269.200 Da (FRAG 1) and 136.100 Da (FRAG 2). These two fragments were formed from acid degradation solution of acalabrutinib during its MS studies and confirm the fragmentation pathway of acid degradant product (DP1) of acalabrutinib. The mass spectra and proposed fragmentation pathway of acid degradant product (DP1) of acalabrutinib is shown in supplementary data as Figure S2(a) and Figure S2(b), respectively.

Mass fragmentation pattern of base degradant product (DP2) of acalabrutinib

In base degradation, when acalabrutinib was reacted with base the terminal acetylene group which is attached to the carbonyl group of acalabrutinib is removed and the terminal methyl group rearrange and directly attached to the carbonyl group leading to the formation of most stable degradant product (DP2) of acalabrutinib whose *m/z* ratio is obtained around 442.100 Da (DP2) and its three protonated fragmentation ion is observed whose m/z ratio is obtained around 330.300 Da (FRAG 1), 198.300 Da (FRAG 2) and 95.300 Da (FRAG 3). These three fragments were formed from base degradation solution of acalabrutinib during its MS studies which could help in the purpose of fragmentation pathway of base degradant product (DP2) of acalabrutinib. The mass spectra and proposed fragmentation pathway of base degradant product (DP2) of acalabrutinib is

shown in supplementary data as Figure S3(a) and Figure S3(b), respectively.

Mass fragmentation pattern of base degradant product (DP3) of acalabrutinib

In base degradation, acalabrutinib on reaction with base undergo nucleophilic addition reaction The nucleophile methoxy obtained from methanol attack on the C≡C system and forms an addition product leading to the formation of most stable degradant product (DP3) of acalabrutinib whose *m/z* ratio is obtained around 498.400 Da (DP3) and its four protonated fragmentation ion is observed whose *m/z* ratio is obtained around 399.200 Da (FRAG 1), 330.300 Da (FRAG 2), 198.300 Da (FRAG 3) and 95.300 Da (FRAG 4). These four fragments were formed from base degradation solution of acalabrutinib during its MS studies which could help in the purpose of fragmentation pathway of base degradant product (DP3) of acalabrutinib. The mass spectra and proposed fragmentation pathway of base degradant product (DP3) of acalabrutinib is shown in supplementary data as Figure S4(a) and Figure S4(b), respectively.

Mass fragmentation pattern of oxidative degradant product (DP4) of acalabrutinib

Hydrogen peroxide causes N-oxide formation over the nitrogen on terminal pyridine ring system in acalabrutinib, leading to the formation of a degradant product of acalabrutinib with an approximate *m/z* ratio of 482.200 Da (DP4) and two protonated fragmentation ions with approximate *m/z* ratios of 372.100 Da (FRAG 1) and 136.100 Da (FRAG 2). These two fragments were generated from the oxidation degradation solution of acalabrutinib in the course of its MS studies with the intention of elucidating the fragmentation mechanism of the oxidative degradant product (DP4). The mass spectra and estimated fragmentation process for DP4, an oxidative degradant product of acalabrutinib, is shown in supplementary data as Figure S5(a) and Figure S5(b), respectively.

Characterization of acid degradation product (DP1) of acalabrutinib by NMR

In acid degradation solution of acalabrutinib, the acid degradant product (DP1) molecule is generated whose *m/z* ratio is obtained around 390.200 Da and its structure is confirmed by ^{1}H NMR as shown in supplementary data in Figure S6.

DP1: MS *m/z* 390.200 [M+H]; ^{1}H NMR: 1.93(3H, s, -CH_3), 2.08-2.13(4H, m, -CH_2), 3.73-3.74(2H, t, -CH_2), 5.83-5.84(1H, t, -CH), 6.98-6.99(1H, d, -Ar H), 7.09-7.10 (1H, d, -Ar H), 7.41(2H, s, -NH_2), 7.76-7.78(2H, m, -Ar H) 7.78-7.80(2H, m, -Ar H), 11.7(1H, s, -OH)

Characterization of base degradation product (DP2 and DP3) of acalabrutinib by NMR

In base degradation solution of acalabrutinib, the base degradant products (DP2 and DP3) molecule is generated whose *m/z* ratio is obtained around 442.100 Da and 498.400 Da and its structure is confirmed by ^{1}H NMR as shown in supplementary data in Figure S7 and Figure S8.

DP2: MS *m/z* 442.100 [M+H]; ^{1}H NMR: 2.10-2.15(4H, m, -CH_2), 2.13(3H, s, -CH_3), 3.79-3.80(2H, t, -CH_2), 5.71-5.72(1H, t, -CH), 6.98-6.99(1H, d, -Ar H), 7.09-7.10(1H, d, -Ar H), 7.19-7.22(1H, m, -Ar H), 7.41(2H, s, -NH_2), 7.77-7.79(2H, d, -Ar H), 7.86-7.89(1H, m, -Ar H), 7.93-7.95(2H, d, -Ar H), 8.36-8.37(1H, d, -Ar H), 8.62-8.63(1H, d, -Ar H), 9.01(1H, s, -NH)

DP3: MS *m/z* 498.400 [M+H]; ^{1}H NMR: 2.08(3H, s, -CH_3), 2.06-2.11(4H,m,-CH_2), 3.75(3H, s, OCH_3), 3.74-3.77(2H, t, -CH_2), 4.96 (1H, s, -CH), 5.75-5.76 (1H,t,-CH), 6.98-6.99 (1H, d, -Ar H), 7.09-7.10 (1H, d, -Ar H), 7.19-7.22 (1H, m,-Ar H), 7.41(2H, s, -NH_2), 7.77-7.79 (2H, d, -Ar H), 7.86-7.89 (1H, m, -Ar H), 7.93-7.95 (2H, d, -Ar H), 8.36-8.37 (1H, d, -Ar H), 8.62-8.63(1H, d, -Ar-H), 9.01(1H, s, -NH).

Characterization of oxidative degradation product (DP4) of acalabrutinib by NMR

In oxidation degradation solution of acalabrutinib, the oxidative degradant product (DP4) molecule is generated whose *m/z* ratio is obtained around 482.200 Da and its structure is confirmed by ^{1}H NMR as shown in supplementary data in Figure S9.

DP4: MS *m/z* 482.200 [M+H]; ^{1}H NMR: 1.93(3H, s, -CH_3), 2.09-2.15(4H, m, -CH_2), 3.74-3.75(2H, t, -CH_2), 5.83-5.84 (1H, t, -CH), 6.94-6.96(1H, m, -Ar H), 6.97-6.99 (1H, d, -Ar H), 7.09-7.10 (1H, d, -Ar H), 7.14-7.15 (1H, m, -Ar H), 7.41(2H, s, -NH_2), 7.46-7.50 (1H, d, -Ar H), 7.57-7.59 (1H, d, -Ar H), 7.77-7.78 (2H, d, -Ar H), 7.92-7.93 (2H, d, -Ar H), 8.96(1H, s, -NH)

CONCLUSION

A new method, LC-MS/MS was validated and developed to quantify acalabrutinib in bulky and in the form of capsule dosage. Specific, simple, accurate, precise, satisfactory and robust methods were developed for obtaining valid data and results. In the industry of pharmaceuticals and laboratories, this developed method will be used for drug analysis. For accessing stability of chemical compounds, studies of forced degradation were used and the stability is the proof of the developing nature of the chromatographic method. The four products of acalabrutinib were isolated. The results of spectral data shows that 390.200 Da is the molecular mass of DP1 and $C_{21}H_{19}N_5O_3$ is molecular formula and chemical name of it is "(S)-4-(8-amino-3-(1-(but-2-ynoyl) pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl)benzoic acid". Two types of impurities were noticed and isolated in the studies of base

degradation. 442.100 Da is the molecular mass of DP2, $C_{24}H_{23}N_7O_2$ is the chemical formula of it and chemical name of it is "(S)-4-(3-(1-acetylpyrrolidin-2-yl)-8-aminoimidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2-yl)benzamide". 498.400 Da is the molecular mass of DP3, $C_{27}H_{27}N_7O_3$ is the chemical formula of it and chemical name of it is "(S)-4-(8-amino-3-(1-(3-methoxybut-2-enoyl) pyrrolidin-2-yl)imidazo[1,5-a]pyrazin-1-yl)-N-(pyridin-2 yl)benzamide". 482.200 Da is the molecular mass of DP4, $C_{26}H_{23}N_7O_3$ is the chemical formula of it and chemical name of it is "2-(4-(8-amino-3-(1-(but-2-ynoyl)pyrrolidin-2-yl) imidazo[1,5-a]pyrazin-1-yl)benzamido)pyridine 1-oxide". Chromatographic method which was developed was valid according to the regulations of ICH and this method could be useful to test stability and quality of acalabrutinib drugs in the pharmaceutical industry.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

LC-MS/MS: Liquid Chromatography Mass Spectrometry; **NMR:** Nuclear Magnetic Resonance; **ICH:** International Conference on Harmonisation; **DPs:** Degradant Products; **MS^n:** Multi Stage Mass Fragmentation Studies; **BTK:** Bruton Tyrosine Kinase; **MCL:** Mantle Cell Lymphoma; **CLL:** Chronic Lymphocytic Leukemia; **RP-HPLC:** Reverse Phase High Performance Liquid Chromatography; **HCOOH:** Formic Acid; **NaOH:** Sodium Hydroxide; **HCl:** Hydrochloric Acid; **MeOH:** Methanol; **ACN:** Acetonitrile; **H_2O_2:** Hydrogen Peroxide; **mg:** Milligram; **mL:** Milliliter; **ESI:** Electrospray Ionization; **CUR:** Curtain Gas; **IS:** Ion spray Voltage; **GS1 and GS2:** Ion source gas; **DP:** Declustering Potential; **FP:** Focusing Potential; **EP:** Entrance Potential; **M:** Molar; **PTFE:** Polytetrafluoroethylene; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **MRM:** Multiple Reaction Monitoring; **R.S.D:** Relative Standard Deviation; **SD:** Standard Deviation; **DP1:** Acid Degradation Product of Acalabrutinib; **DP2:** Base Degradation Product of Acalabrutinib; **DP3:** Base Degradation Product of Acalabrutinib; **DP4:** Oxidation Degradation Product of Acalabrutinib; **FRAG 1:** Fragment 1; **FRAG 2:** Fragment 2; **FRAG 3:** Fragment 3; **FRAG 4:** Fragment 4; **^{1}H NMR:** Proton Nuclear Magnetic Resonance.

SUMMARY

Stability indicating LC-MS/MS method was developed and validated as per ICH guidelines. A forced degradation study was carried out on acalabrutinib drug under acidic, basic, neutral, oxidative, photolytic and thermal stress conditions. A total of four degradation products of acalabrutinib was identified and characterized by LC-MS/MS techniques. All the four degradation products of acalabrutinib were isolated through the preparative HPLC system. ^{1}H-NMR studies was also conducted for confirmation of degradation products of acalabrutinib drug.

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Supplementary Data

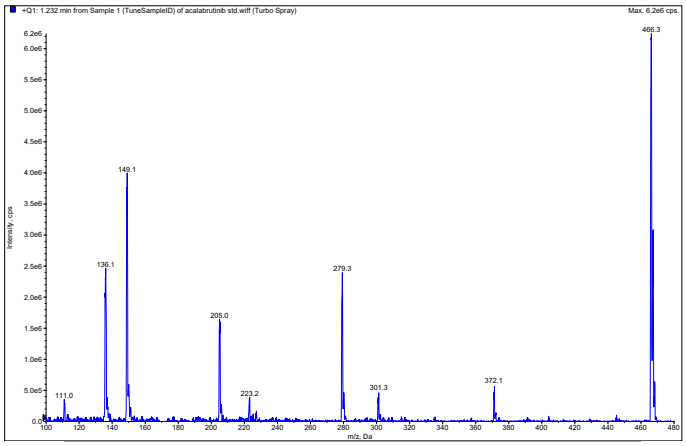

Figure S1:(a) Mass spectra of acalabrutinib.

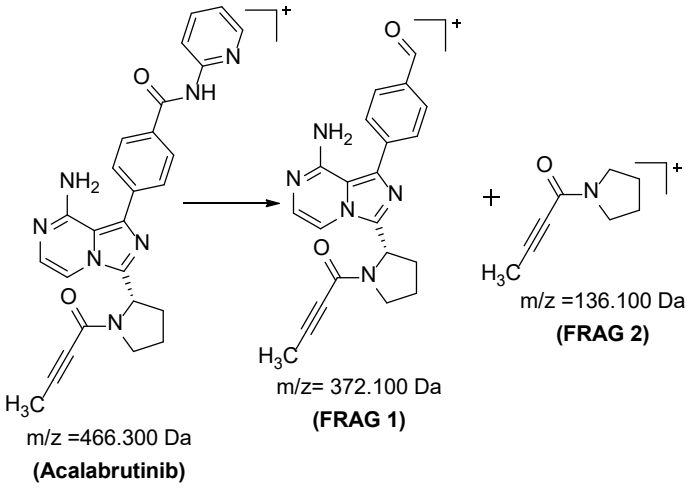

Figure S1: (b) Proposed fragmentation pathway of acalabrutinib.

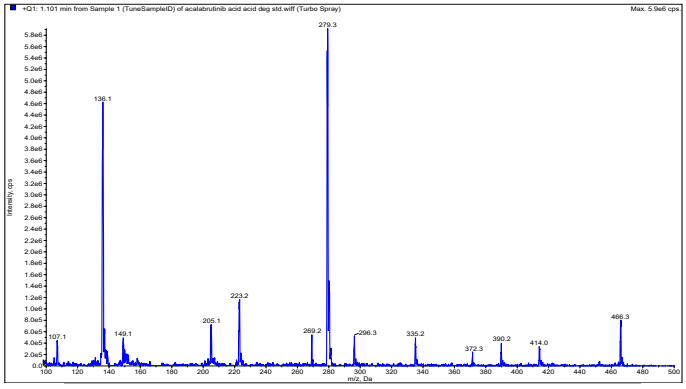

Figure S2: (a) Mass spectra of acid degradant product (DP1) of acalabrutinib.

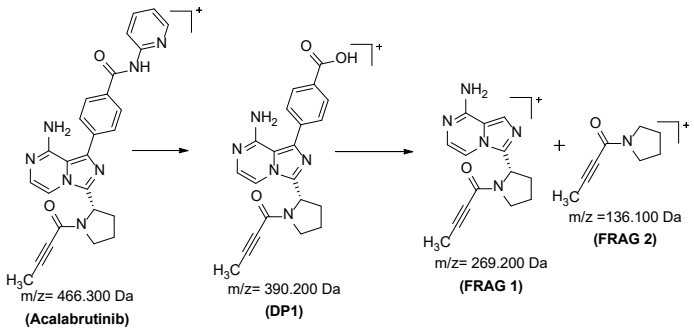

Figure S2: (b) Proposed fragmentation pathway of acid degradant product (DP1) of acalabrutinib.

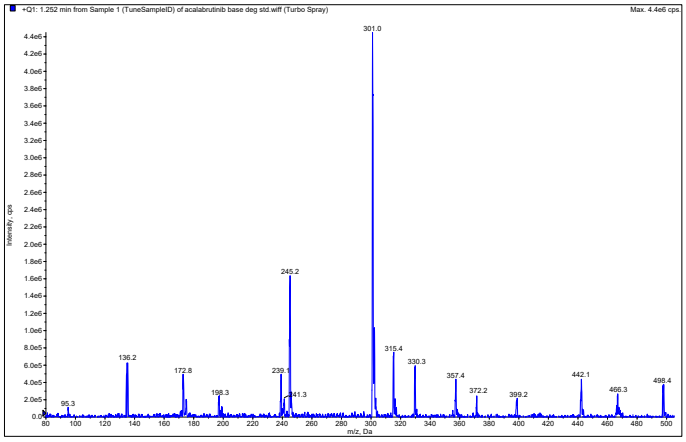

Figure S3: (a) Mass spectra of base degradant product (DP2) of acalabrutinib.

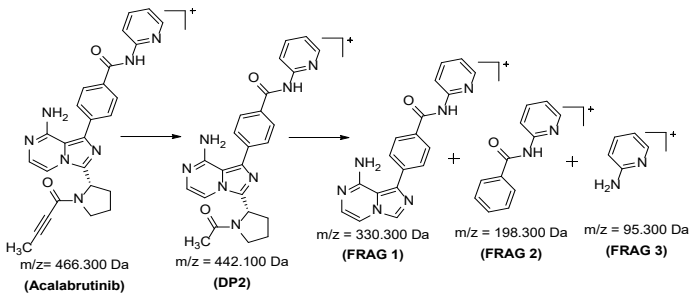

Figure S3: (b) Proposed fragmentation pathway of base degradant product (DP2) of acalabrutinib.

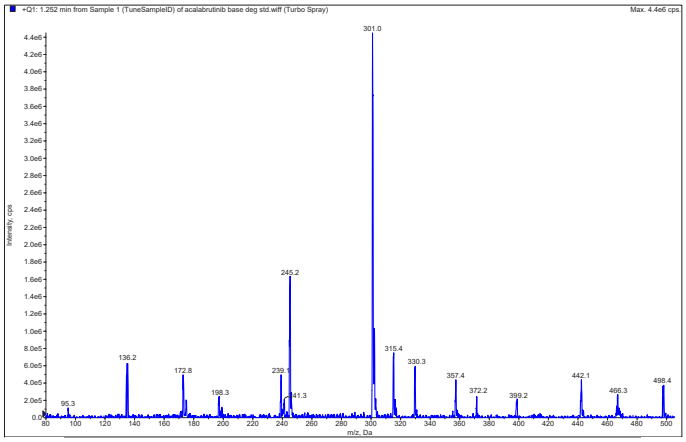

Figure S4: (a) Mass spectra of base degradant product (DP3) of acalabrutinib.

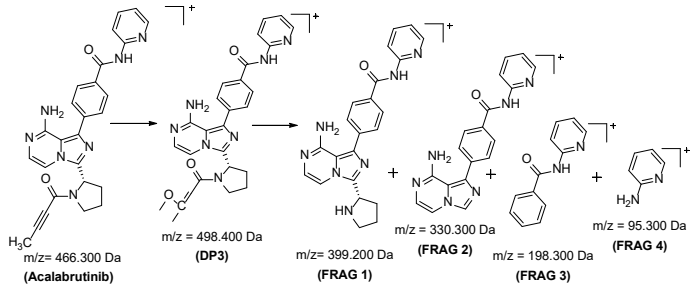

Figure S4: (b) Proposed fragmentation pathway of base degradant product (DP3) of acalabrutinib.

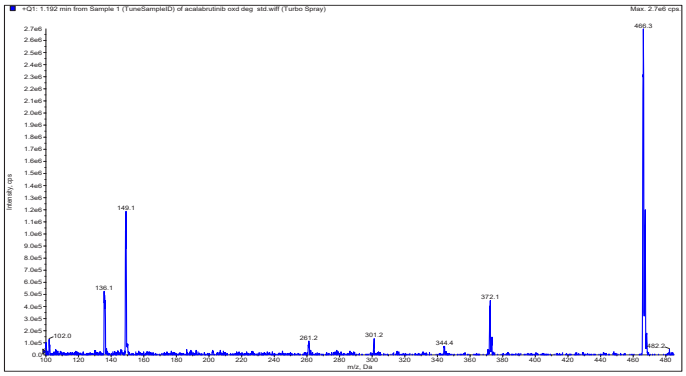

Figure S5: (a) Mass spectra of oxidative degradant product (DP4) of acalabrutinib.

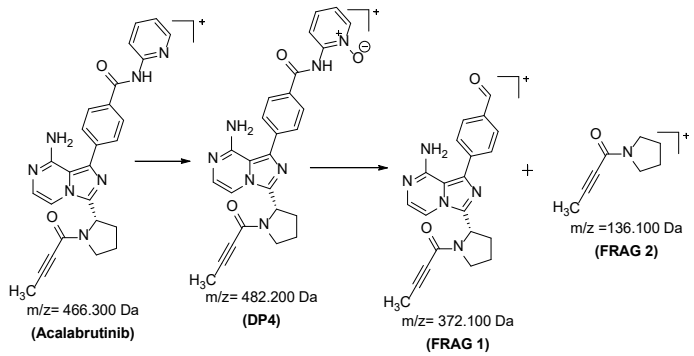

Figure S5: (b) Proposed fragmentation pathway of oxidative degradant product (DP4) of acalabrutinib.

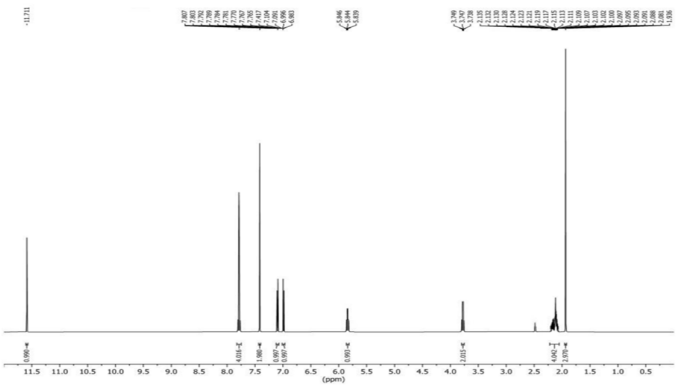

Figure 6: ^{1}H-NMR spectra of acid degradant product (DP1) of acalabrutinib.

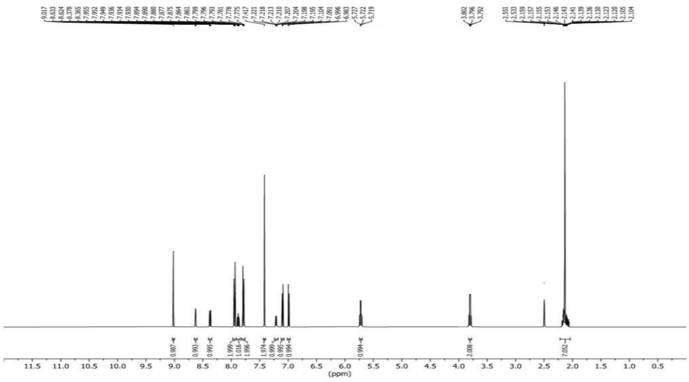

Figure 7: ^{1}H-NMR spectra of base degradant product (DP2) of acalabrutinib.

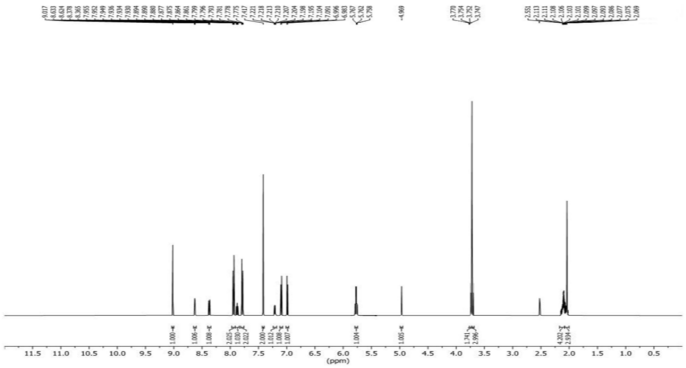

Figure 8: ^{1}H-NMR spectra of base degradant product (DP3) of acalabrutinib.

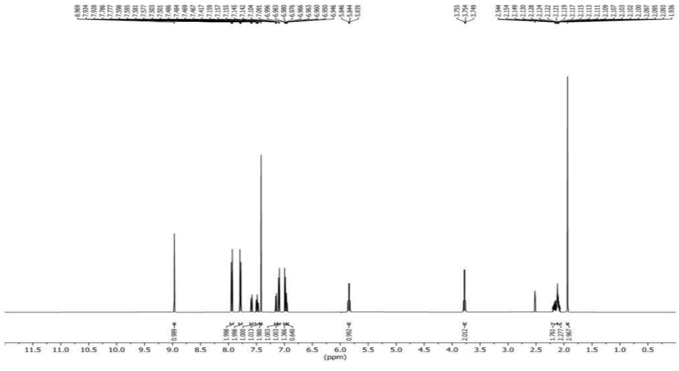

Figure 9: ^{1}H-NMR spectra of oxidative degradant product (DP4) of acalabrutinib.