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A QUALITY BY DESIGN APPROACH FOR DEVELOPMENT OF SIMPLE AND ROBUST REVERSED PHASE STABILITY INDICATING HPLC METHOD FOR ESTIMATION OF IBRUTINIB AND ITS IMPURITIES

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ABSTRACT
Novel stability-indicating quality by design-based reversed-phase HPLC method is developed for the determination of Ibrutinib capsules and its impurities. A column (X-Bridge-C18 150 x 4.6 mm, 3µm,) with the stable bond stationary phase and two different mobile phases A and B are used for effective separation of Ibrutinib and its impurities. All compounds are monitored using a Photo Diode Array detector at 220 nm. The developed technique is found to be vigorous within the distinct design space and the flow gradient has been optimized. Ibrutinib is degraded under various stress test conditions as per International Council for Harmonisation and the parameters namely; linearity, stability, specificity, accuracy, precision, limit of detection and limit of quantization are assessed. To achieve the limit of quantization values under the reporting threshold, injection volumes and test concentrations have been optimized. The developed methodology is successful and robust and supportive in the areas where regulatory agencies propose HPLC analytical techniques.
Keywords: HPLC-PDA, Analytical Quality by Design, Experimental Design, Ibrutinib, Method Validation.
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INTRODUCTION
Ibrutinib is chemically designated as 1-[(3R)-3-[4-amino-3-(4-phenoxy phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one. The molecular weight and formula of Ibrutinib are 440.51 g/mol and $C_{25}H_{24}N_6O_2$ respectively. Ibrutinib belongs to a class of Tyrosine Kinase inhibitors for the remedy of B-cell malignancies. Ibrutinib is approved by the US FDA in 2014, for treatment of B-cell cancers like Waldenstrom's Macroglobulinemia (WM)[1], mantle cell lymphoma[2], and chronic lymphocytic leukemia[3]**,** because of Burton's tyrosine kinase (BTK) protein, which is important in B cells is covalently bound the Ibrutinib drug. Basically, Ibrutinib works by stopping or slowing the escalation of cancer cells. The oral bio-availability of Ibrutinib is 15.9% after consumption of grapefruit juice, 8.4% in a fed state, and 3.9% in a fasting state.[4]

In the recent past, HPLC becomes a conventional analytical technique because of its benefits of high simple throughput and low operating cost. The advantage of this technique is, different samples can be auto-injected into the system and run in a pre-programmed series using a small quantity of the mobile phase. Hence, this technique was adopted and validated by analytical quality by design (AQbD) approach with two different mobile phases for rapid separation of impurities from the drug formulations of Ibrutinib. The method is comprehensively validated as per the guidelines of the International Conference on Harmonisation.[5-8]

Few techniques have been reported for the estimation of Ibrutinib, either alone or with other drugs combination, such as LC-MS/MS[9], LC-TMS[10], RP-HPLC[11-14] and Ultra HPLC[15]. Further, the RP-HPLC technique is extensively used nowadays for the determination of a few vital drugs.[16-19] However, all known and unknown impurities for Ibrutinib in the dosage formulations are not estimated by the reported

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analytical methods. Hence, attempted to authenticate and develop a sensitive, precise, and simple stability indicating RP-HPLC technique for the estimation of Ibrutinib and its impurities in the drug product. This paper represents the quantification of all impurities of Ibrutinib namely IBT4A, IBT6A, Acetyl impurity, Diacryloyl impurity, Chloro impurity, IBT6A Ibrutinib dimer, Thermal degradation dimer, Ibrutinib dimer, IBT6A adduct and IBT7A adduct in Ibrutinib capsules. The molecular structures and IUPAC names of Ibrutinib and its impurities are presented in Fig.-1 and Table-1 respectively.

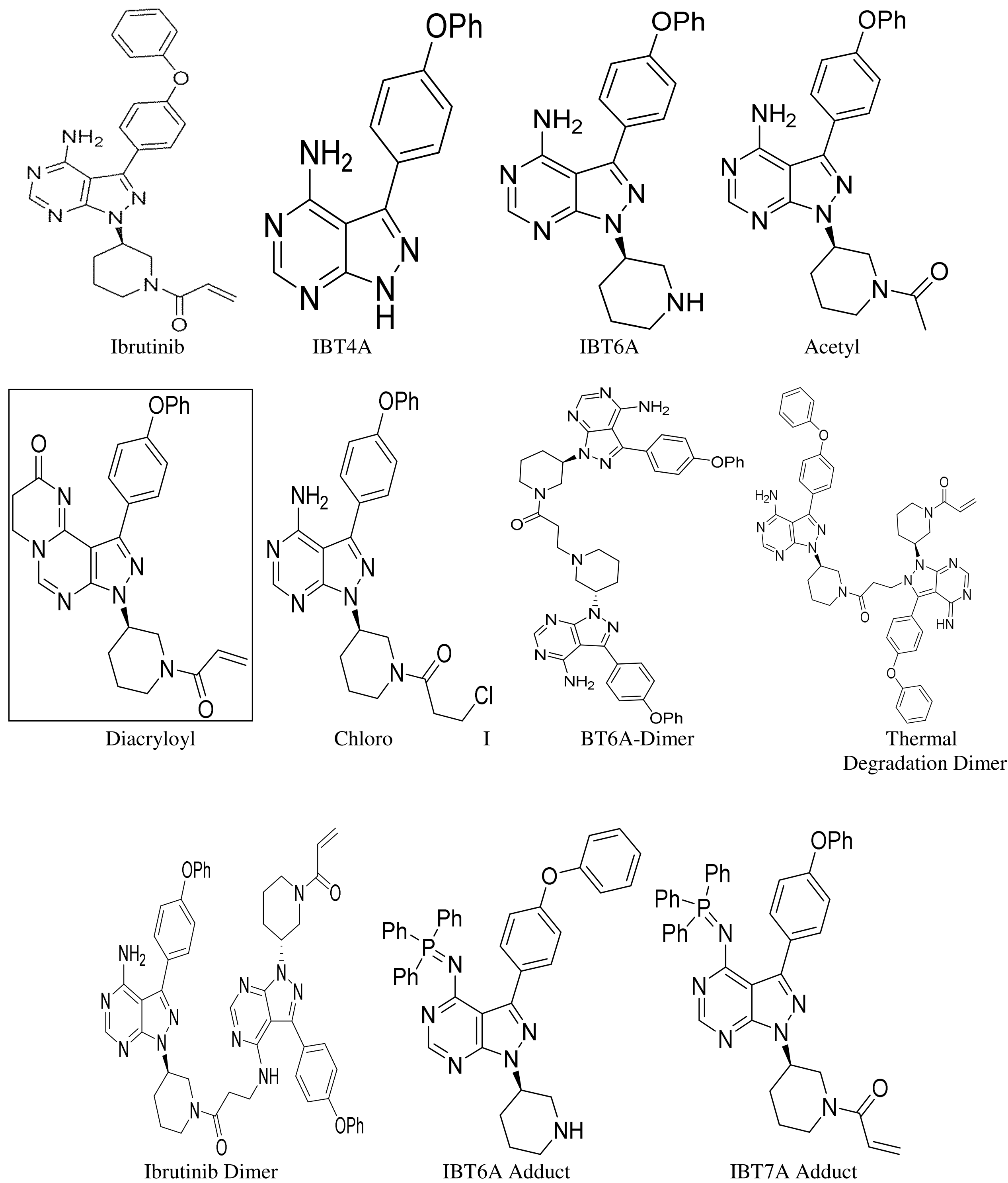

Fig.-1: Molecular Structures of Ibrutinib and its Impurities

Table-1: IUPAC Names, Molecular Formula and Weights of Ibrutinib Impurities

S. No	Impurity Name	IUPAC Names	Molecular Formula	Molecular Weight
1	IBT4A	3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine	$C_{22}H_{22}N_6O$	303.33
2	IBT6A	(R)-3-(4-phenoxyphenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine	$C_{22}H_{22}N_6O$	386.46
3	Acetyl impurity	(R)-1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)ethan-1-one	$C_{24}H_{24}N_6O_2$	428.50
4	Diacryloyl impurity	(*R*)-8-(1-acryloylpiperidin-3-yl)-10-(4-phenoxyphenyl)-3,4-dihydropyrazolo[4,3-e]pyrimido[1,2-c]pyrimidin-2(8H)-one	$C_{28}H_{26}N_6O_3$	494.56
5	Chloro impurity	(R)-1-(3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-chloropropan-1-one	$C_{25}H_{25}ClN_6O_2$	476.97
6	IBT6A Ibrutinib dimer	1-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-((S)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)propan-1-one	$C_{47}H_{46}N_{12}O_3$	826.97
7	Thermal degradation dimer	1-((S)-3-(2-(3-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropyl)-4-imino-3-(4-phenoxyphenyl)-2,4-dihydro-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one	$C_{50}H_{48}N_{12}O_4$	880.39
8	Ibrutinib dimer	1-((R)-3-(4-((3-((R)-3-(4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)-3-oxopropyl)amino)-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl)piperidin-1-yl)prop-2-en-1-one	$C_{50}H_{48}N_{12}O_4$	881.01
9	IBT6A adduct	R)-N-(3-(4-phenoxyphenyl)-1-(piperidin-3-yl)-1H-pyrazolo[3,4-d]pyrimidin-4-yl)-1,1,1-triphenyl-l5-phosphanimine	$C_{40}H_{35}N_6OP$	646.26
10	IBT7A adduct	(R)-1-(3-(3-(4-phenoxyphenyl)-4-((triphenyl-l5-phosphaneylidene) amino)-1H-pyrazolo [3, 4-d] pyrimidin-1-yl) piperidin-1-yl) prop-2-en-1-one	$C_{43}H_{37}N_6O_2P$	700.78

EXPERIMENTAL

Equipment and Chemicals

The Waters High-performance liquid chromatography (HPLC) instrument with Photodiode Array detector was used for developing and validating. Waters Empower Networking Software was used for evaluating the peak purity of Ibrutinib and all impurities. The Ibrutinib capsules (purity, ≥ 99.9%) and its impurities were supplied by Dr. Reddy’s Laboratories Limited Hyderabad, India. The Thermo Fisher Scientific

electronic analytical balance was employed for weighing functions. All the chemicals namely Trifluoroacetic acid, Potassium dihydrogen phosphate, Potassium hydroxide, Methanol and Acetonitrile used were analytical reagent grade chemicals and purchased from Merck. India Pvt. Ltd.

Chromatographic Conditions

The method development and validation of the samples have been done by using reversed-phase HPLC instrument with a Photodiode array detector. A stationary phase was developed with the C18 (X Bridge C18) column with 150 mm length, 4.6 mm internal diameter, and 3.5μ particle size and all impurities were separated by adopting a gradient flow technique. The column temperature and sample cooler temperatures were maintained at 40º C and 5°C respectively and all impurities were monitored at 220 nm. The 10 μL samples were injected into the HPLC system by maintaining the flow rate of 1.0 mL/min in the mobile phase. Methanol was used to the preparation of diluents extraction of Ibrutinib and its impurities from the formulation matrix.

Preparation of Buffer-1 and Buffer-2 Solutions

Accurately weighed 1.36 g of potassium dihydrogen phosphate (KH_2PO_4) and 0.5 mL of Trifluoroacetic acid (TFA) were added into a 2.0 L volumetric flask, diluted to the mark using Milli Q water with occasional shaking and the pH was adjusted to 5.5 using Potassium Hydroxide (KOH). Thus the prepared solution was used as buffer-1. To prepare buffer-2, 1.36 g of KH_2PO_4 along with 0.7 mL of TFA was transferred into a 1.0 L volumetric flask and diluted to the mark using Milli Q water and pH was adjusted to 5.5 using KOH.

Preparation of Mobile Phase-A and Mobile Phase-B

Mobile phase-A and mobile phase-B were prepared by mixing the volumes of buffer and Acetonitrile solutions in the ratio of 85:15 and 30:70 respectively. Mobile phase-A contains additional buffer concentration than mobile phase-B to divide all the impurities and mobile phase-B consists of additional organic concentration than mobile phase-A to elute all the impurities.

Standard Ibrutinib Solution Preparation

Ibrutinib stock solution was prepared by adding 50 mg of Ibrutinib standard into a 100 mL volumetric flask which contained a 70 mL of diluent and the contents were kept on sonicator until absolute digestion of the drug. Then the mixture was diluted up to the mark using diluent and mixed properly. Further, 4.0 mL of above-prepared stock solution was transferred into a 50 mL flask and diluted the volume with diluent. Thus obtained solution was used as a working Ibrutinib solution of 2 mg/L concentration in the present work.

Preparation of Sample

Accurately weighed and transferred 100 mg of Ibrutinib capsule powder into a 100 mL flask and to it, 70 mL of diluents was added and sonicated for 30 min with intermediate shaking in the ice water bath. The flask was then kept on the bench top to reach the room temperature and makeup to the volume with diluent and mixed well. Then, the resultant solution was centrifuged for 5 min at 4000 RPM.

Stock Impurity Preparation

2 mg of each impurity was accurately weighed and transferred into 20 mL volumetric flasks containing 5 mL of methanol in each flask. Then, the mixture of methanol and impurities in the flasks were sonicated until complete digestion of the impurities and diluted to the mark using diluent.

Spiked Sample Preparation

100 mg of Ibrutinib tablet powder was accurately weighed and transferred into a 100 mL volumetric flask and to it, 70 mL of the diluent was added. 1.25mL of stock impurity solution was added to the flask. Then, the resultant solution was sonicated for 30 minutes with intermittent shaking in the presence of ice.

The flask was then kept on the bench top to acquire the room temperature and makeup to the volume with diluent and mixed properly. Further, the solution was centrifuged for 5 minutes at 4000 RPM.

Selectivity

In the occurrence of Ibrutinib degradation products, the selectivity of the present developed HPLC method for Ibrutinib has been carried out[5-8]. To provide an identification of the specificity of the present method, the stress studies were performed for the drug Ibrutinib. Separate portions of Ibrutinib samples were exposed to stress circumstances like acid (2N HCl), base (1N NaOH), peroxide (30% H_2O_2), water (water bath), thermal (hot air oven), humidity (humidity chamber at 90%) and photolytic (photo stability chamber). The Ibrutinib and mixture of its impurities were injected into the optimized system to demonstrate the specificity of the developed method in the formulation of Ibrutinib.

RESULTS AND DISCUSSION

Chromatographic Conditions Optimization

Table-2: CMP, CQA, and QTMP of IBTA related Substance Stability-Indicating Analysis Method

Critical Method Parameter	Range of each parameter used for DOE			Quality Target Method Profile	Critical Quality Attribute
	Low	As such	High	Targeted QTMP	
A. pH of M.P-A	5.3	5.5	5.7	Resolution b/w Acetyl and Diacryloyl not less than-1.5	Resolution b/w Acetyl and Diacryloyl
B. %ACN in M.P-B	63	70	77		
C. Column temp	35	40	45		
D. pH of M.P-B	5.3	5.5	5.7		

To get the best resolution amid Ibrutinib and its impurities, several mobile phase compositions and different stationary phases were investigated in the preliminary studies. All analytes have different retention behaviors and hence it is a challenging development to separate all analytes in the shorter method without interfering Placebo components and degradation impurities. The final chromatographic conditions were optimized based on the Design of Experiments (DOE) which gives an influential site for a statistical methodology and the obtained results are presented in Table-2. The DOE was carried out employing fractional design by considering the flow rate, buffer solution pH and resolution amid the close eluting impurities (Impurity-B&H) as responses. The factors of 10 combinations have been used and the results are depicted in Table-3.

Table-3: Design of Experiments runs –IBTA related Substance Method

Std Order	Run Order	Centre Point	pH of M.P-A	%ACN in M.P-B	Column temp	pH of M.P-B	Resolution b/w Acetyl and Diacryloyl
7	1	1	5.3	77	45	5.3	1.7
3	2	1	5.3	77	35	5.7	1.7
4	3	1	5.7	77	35	5.3	1.2
1	4	1	5.3	63	35	5.3	1.9
5	5	1	5.3	63	45	5.7	1.9
8	6	1	5.7	77	45	5.7	1.1
2	7	1	5.7	63	35	5.7	1.6
6	8	1	5.7	63	45	5.3	1.5
9	9	0	5.5	70	40	5.5	1.8

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Minitab software was employed for evaluating the effects of parameters on resolutions and to generate the Pareto chart with three-dimensional plots. Buffer solution pH in mobile phase-A and percentage of acetonitrile in mobile phase-B plays a significant role in the separation of impurities. Further, the obtained data were used for setting the lower and upper boundaries for each variable. Moreover, the design space was demonstrated and experimentally suggested findings were closer to the proposed factors. By using modeled data and visual inspections, different overlay graphs were plotted between two parameters at a time. The parameters of the developed and validated HPLC method are presented in Table-4. The main effect chart, interaction plots, Pareto charts for standardized effects and contour plot for resolution between Acety and Diacryloyl impurities are presented in Fig.-2 to Fig.-5 respectively.

Table-4: Optimized HPLC Method Conditions

Column	X-Bridge-C18 150 x 4.6 mm, 3. µm		
Flow rate	1.0 mL /min		
Column oven temperature	40 °C		
Wave length	220 nm		
Injection Volume	10 µL		
Run time	65 minutes		
Gradient Program	Time (min)	% Mobile Phase-A	% Mobile Phase-B
	0	65	35
	8	55	45
	23	45	55
	43	10	90
	48	0	100
	58	0	100
	59	65	35
	65	65	35

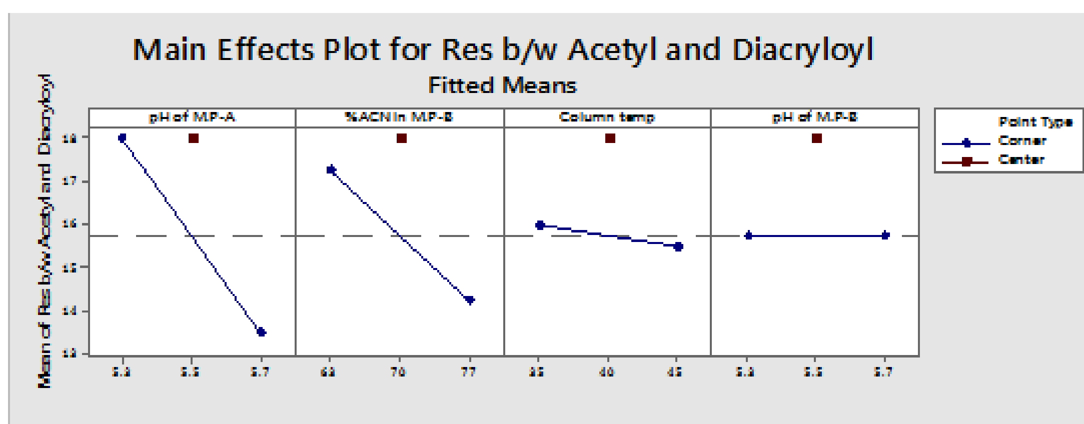

Fig.-2: The Main Effect Chart for the Resolution between Acetyl and Diacryloyl Impurities

The diluents were confirmed based on the recovery and shape of the peak and injection volumes and test sample concentrations were get high reporting threshold than the limit of quantification. The gradient was optimized to get the best resolution among the main analyte and all impurities. The chromatograms of blank and standard solutions are presented in Fig.-6 and Fig.-7.

Suitability of the System

The prepared Ibrutinib test solutions of standard levels were aspirated into the HPLC system and found that system suitability parameters are within the limits. The relative standard deviation (RSD) percentage

was evaluated for USP plate count and peak areas repeatedly. The replicated injection of the percentage of RSD was found as 0.7 % where the acceptance criteria were not more than 10.0%.

Stability of the Solution

The stability of the Ibrutinib and its impurities in the spiked sample was investigated for 24 h at room temperature on the bench-top. System suitability parameters and percentages of impurities were assessed and aligned with a fresh standard. It is found that the solutions and mobile phases steady up to 24 h as all spiked samples were kept in the air-tight flasks.

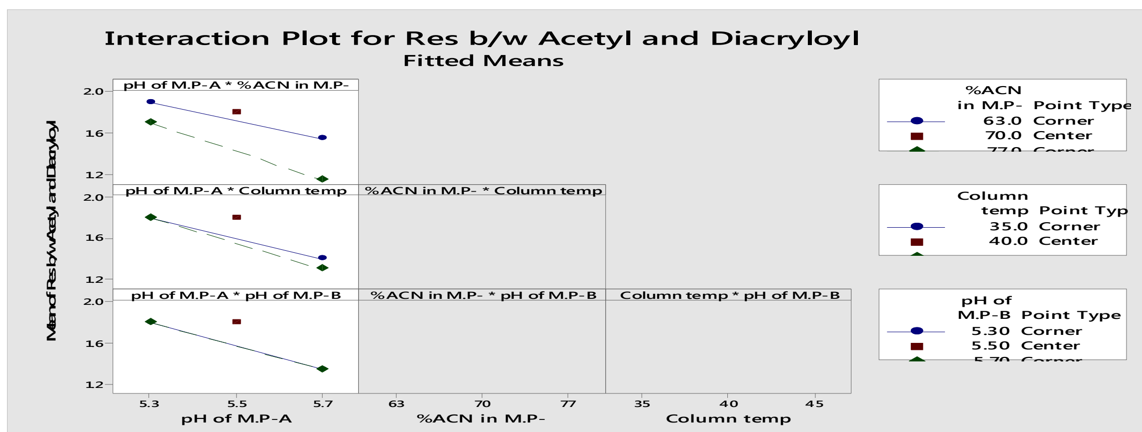

Fig.-3 The Interaction Plot for the Resolution between Acetyl and Diacryloyl Impurities

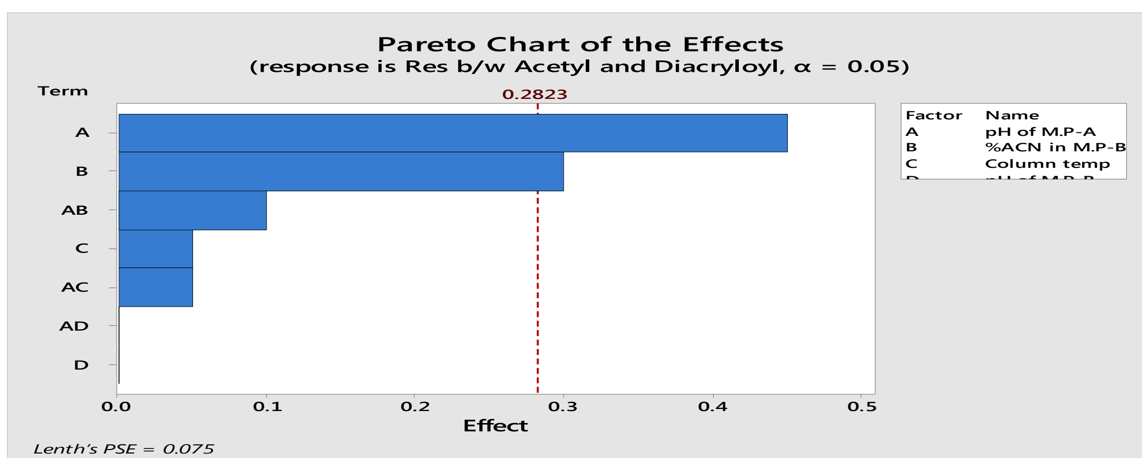

Fig.-4: The Pareto Chart for Standardized Effects on the Resolution between Acetyl and Diacryloyl impurities

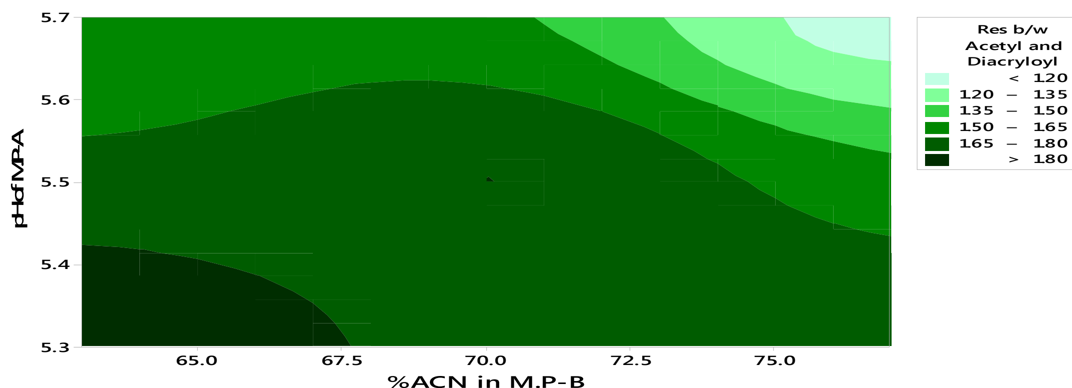

Fig.-5: The Contour Plot for the Resolution between Acetyl and Diacryloyl Impurities

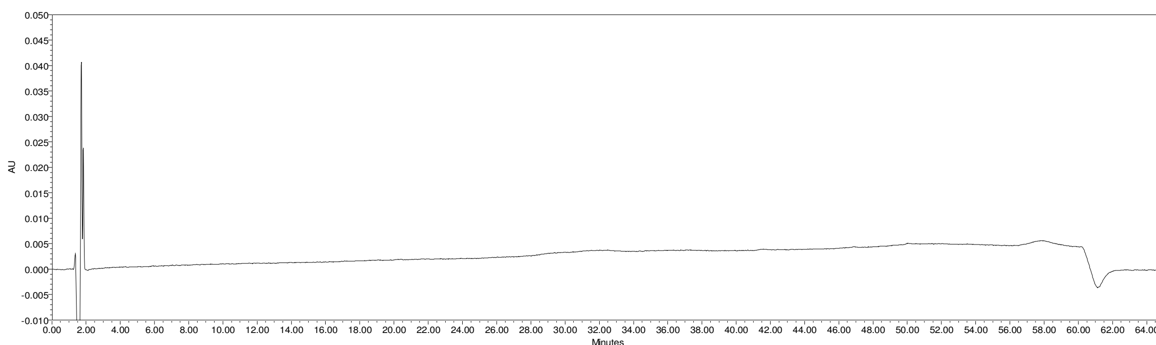

Fig.-6: Typical Chromatogram of the Blank Solution

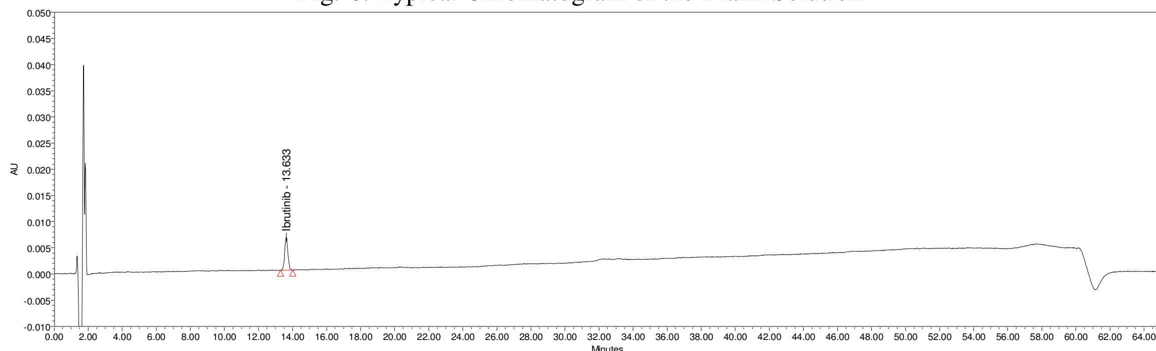

Fig.-7A: Typical Chromatogram of Standard Solution

Specificity

(i) Placebo Interference

A study was conducted to establish the interference of placebo interference. As per the test method, samples were prepared by taking the placebo and then injected into the HPLC system. Interference was not found for the chromatograms of placebo solution, empty cell solution and impurities solution at the retention time of Ibrutinib and its impurities. The obtained chromatogram is presented in Fig.-8.

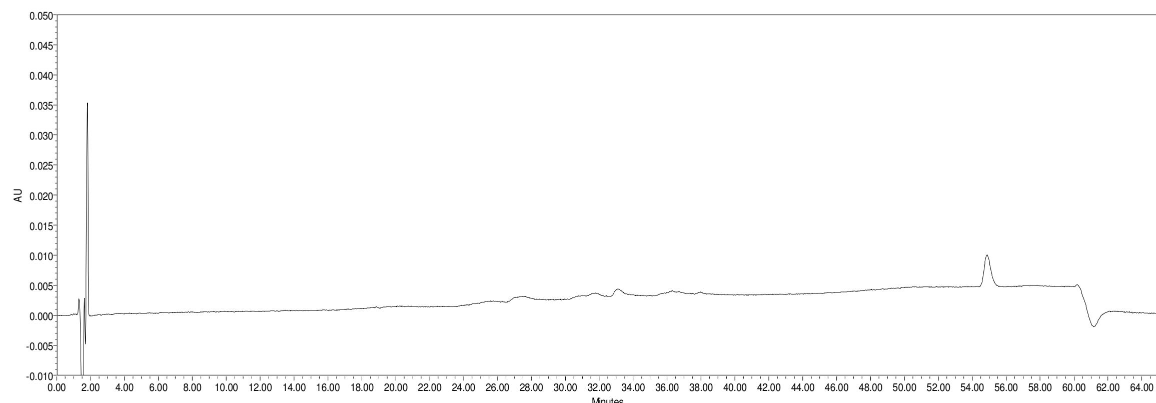

Fig.-8: Typical Chromatogram of Placebo

(ii) Interference from Degradation products

The samples were subjected to diverse stress circumstances for the efficient separation of all degradants from the forced degradation of Ibrutinib. Separate portions of Ibrutinib capsules were exhibited to subsequent stress conditions to induce degradation and the detailed findings are depicted in Table-5.

A. Acid Degradation- Kept on a water bath at 50°C for 2 h with 2N HCl
B. Base Degradation- Kept on bench top for 2 h with 1N NaOH.
C. Peroxide Degradation - Kept on the benchtop in dark for 24 h with 30% H_2O_2 solution.
D. Water Degradation- Kept on a water bath at 50°C for 4 h.
E. Thermal Degradation - Kept in a Hot-air oven at 105° for 24 h.

F. Humidity Degradation - Kept in Humidity chamber at 90% RH for 5 days at 25°C.
G. Photolytic Degradation - Exposed to 200-watt h/m^2 and 1.2 million lux hours in Photo stability chamber for 16 h.

Table-5: Interference from Degradation Products

S. No.	Stress Condition	% Net Degradation (% imp of the Stressed Sample - % imp of the Unstressed Sample)	Purity Angle	Purity Threshold	Purity Flag (Yes/No)
1	Unstressed sample	0.36	0.068	0.272	No
2	2N HCl_5mL_for 2 Hrs on at 50°C	3.58	0.071	0.272	No
3	1N NaOH_5mL_for 2 Hrs on benchtop	4.11	0.046	0.265	No
4	30% hydrogen peroxide_10mL_for 72 Hrs on benchtop	0.10	0.063	0.272	No
5	Exposed to 200 watt hour/m^2 and 1.2 million lux hour in Photostability chamber for 16Hrs	0.0	0.062	0.268	No
6	Heat at 105°C for 24 Hrs	2.2	0.063	0.266	No
7	Humidity at 90% RH for 5 days at 25°C	0.0	0.056	0.265	No
8	Water_10mL for 4hrs at 50°C	0.0	0.061	0.272	No

All the stressed samples were subjected to HPLC system to find the impurity peaks and purity of the main analyte. Impurity degradant peaks in chromatograms of all stressed samples and Ibrutinib were resolved. Using Waters Empower Networking Software, stressed samples chromatograms were assessed for peak purity of Ibrutinib and all impurities. The purity angle was found fewer than the purity threshold for all forced degradation samples. This shows that there is no interference and co-elution from degradants in the quantification of impurity in the drug product. Hence, this method is "Stability Indicating" and extremely specific. The assay and mass balance of degradation samples are presented in Table-6.

Table-6: Mass Balance of Degradation Sample

S. No.	Stress Condition	% Degradation	%Assay	% Mass Balance
1	Unstressed Sample	0.36	95.5	-
2	2N HCl_5mL_for 2 Hrs on at 50°C	3.58	91.6	99.3
3	1N NaOH_5mL_for 2 Hrs on benchtop	4.11	85.5	93.5
4	30% hydrogen peroxide_10mL_for 72 Hrs on benchtop	0.10	92.9	97.0
5	Exposed to 200-watt hour/m^2 and 1.2 million lux hour in Photostability chamber for 16Hrs	0.0	95.8	99.5
6	Heat at 105°C for 24 Hrs	2.2	94.7	101.1
7	Humidity at 90% RH for 5 days at 25°C	0.0	95.1	99.2
8	Water_10mL for 4hrs at 50°C	0.0	98.4	102.6

LOD and LOQ

Table-7: LOD, LOQ. Linearity, Precision and Accuracy Values

Parameters	Ibrutinib	IBT6A	Acetyl	Diacryloyl	Chloro	Dimer-1
LOQ(%)	0.038	0.035	0.037	0.034	0.041	0.029
LOD(%)	0.012	0.011	0.014	0.012	0.013	0.010
Precision % RSD	1.7	1.2	1.0	0.9	0.9	1.7
Accuracy						
LOQ	108.4	104.2	105.4	105.6	104.2	94.0
100%	104.9	100.9	106.8	103.3	101.3	110.3
150%	104.8	103.6	103.9	104.4	102.9	109.4

To investigate the LOQ and LOD, dissimilar concentrations of solutions were prepared by spiking known amounts of Ibrutinib and its impurities with the diluents. The equations LOQ=10×σ/S and LOD=3.3×σ/S (where, σ is the standard deviation of the response and S is the calibration curve slope) were used for the estimation of LOQ and LOD values by the slope method. LOD values were found between 0.010-0.014 and LOQ values were found between 0.029-0.041. By the signal-to-noise (S/N) approach, quantitation limits and detection limits were established and the concentration ratio with a signal to the noise about three was taken as LOD and ten was taken as LOQ. The obtained findings were depicted in Table-7.

Linearity and Range

The linearity was investigated in the range of 0.3 mg/L to 4.0 mg/L for all impurities and for the main analyte. The six prepared dissimilar linearity solutions were injected into the HPLC system, and the obtained results are depicted in Table-7.

Accuracy and Precision

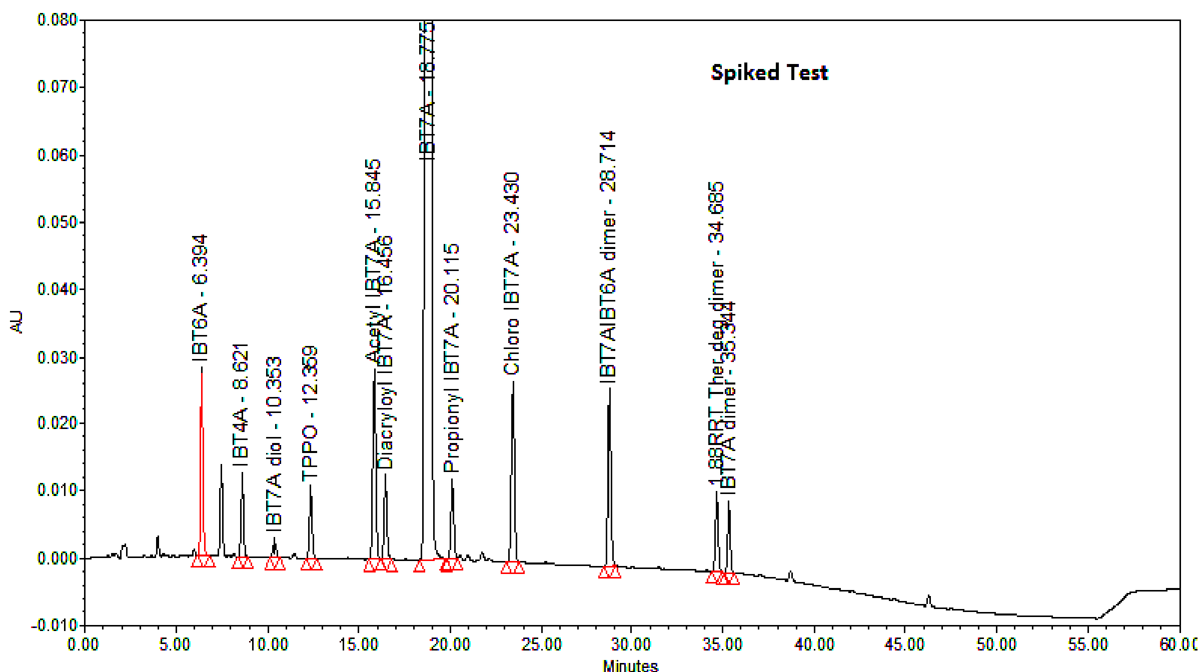

Fig.-9: Typical Chromatogram of Spiked Test

Six samples of 0.2% targeted test solutions were prepared at by spiking the Ibrutinib impurities. The obtained chromatogram is presented in Fig.-9. The precision was calculated within different runs (inter-day) and a single run (intra-day). The accuracy was evaluated as %bias (divergence amid nominal

concentrations and measured concentrations). Recovery studies from 0.3 mg/L to 4.0 mg/L were performed for all impurities, and the values were obtained between 94-108.4 %. The developed method is precise and accurate as the ensuing percentage of RSD (Relative Standard Deviation) values of Everolimus impurities were observed below 2.0 (n=6). The acquired results are depicted in Table-7.

CONCLUSION

A novel and simultaneous stability-indicating QbD-based RP-HPLC technique for the estimation of Ibrutinib impurities were developed with a column (X-Bridge-C18 150 x 4.6 mm, 3µm,) containing a stable bond stationary phase. The present developed technique was validated by testing its accuracy, specificity, precision, linearity, stability, LOD and LOQ. The developed technique is found to be specific and stability-indicating and shows an outstanding performance in terms of speed and sensitivity. Samples are subjected to different forced degradation studies and found that impurity degradant peaks in chromatograms of all stressed samples and Ibrutinib are resolved. Recoveries studies are performed from 0.3 mg/L to 4.0 mg/L for all the impurities, and the values were obtained between 94-108.4 %. The developed technique is found to be vigorous within the distinct design space. Hence, this method can be applied for assessing the impurities in Ibrutinib finished dosage forms. Further, conventional reported HPLC methods may be replaced by the proposed HPLC method because of its superiority.

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