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Original Article

A STABILITY, ACCURACY, AND ROBUSTNESS REPRESENTING LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTIFICATION OF ZANUBRUTINIB AND ITS SPECIFIED IMPURITIES

SUBHASHINI KANTHETI[1*], R. RAMESH RAJU[2]

[1]Department of PG Chemistry, Akkineni Nageswara Rao College, Gudivada-521301, India. [2]Department of Chemistry, Acharya Nagarjuna University, Nagarjuna Naga, Andhra Pradesh, India

[*]Corresponding author: Subhashini Kantheti; [*]Email: subhashinikantheti@gmail.com

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ABSTRACT

Objective: An innovative RP-HPLC isocratic method was established and then validated using Zanubrutinib and its specified impurities (Impurity-1, Impurity-2, Impurity-3, Impurity-4, and Impurity-5).

Methods: In this method, effective chromatographic separation was given an X-Bridge Phenyl column measuring 250 mm x 4.6 mm, packed column with 5µ as a particle size. Acetonitrile, 1% Ortho Phosphoric acid (pH: 2.7), and methanol in the volume ratios 40, 40, and 20 were utilized as a mobile phase at room temperature with an optimized 1.0 ml/min flow rate. Wavelength was detected at 225 nm by using a PDA detector.

Results: Retention times of zanubrutinib and its specified impurities were recorded at 13.284, 4.730, 6.816, 9.583, 10.726, and 12.287. Moreover, other parameters USP tailing is good, USP plate count above 4000, and USP resolution is greater than are equal to 2. The Obtained peaks are homogeneous, hence the purity angle is less than the purity threshold and No Purity Flag. According to ICH guidelines, this method was validated. Zanubrutinib (5-75 µg/ml), their quantified impurity-1, impurity-2, impurity-4, impurity-5 (0.1-1.5 µg/ml), and impurity-3 (0.1-1.5 µg/ml) are proved through linearity method in between LOQ to 75 quantified levels. The % recovery was present between 100.18-95.85, 103.15-93.80, which is a good and acceptance range (amongst 85% and 115%) for drug and specified impurities. The limit of quantitation (LOQ) and limit of detection (LOD) values were assessed for zanubrutinib and its specified impurities were tabulated. These values were calculated using slope (σ) and standard deviation (SD) methods. Method precision (M. P.) and Intermediate (I. P.) Intermediate (I. P.) precision was estimated by evaluating several (six) samples of a similar batch as per the planned technique on the day and the next day, using different columns and systems. Robustness information significantly affects the resolution between Zanubrutinib and specified impurities. The remaining parameters do not impact the parameter's system suitability.

Conclusion: Hence this method was chosen for common analysis. Finally, the system-suitable parameters and validation parameters values are acceptable limits.

Keywords: Zanubrutinib**,** Specified impurities, Linearity recovery, and robustness

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INTRODUCTION

A Zanubrutinib (ZBB/BGB-3111) is classified as a (BTK) Bruton tyrosine kinase inhibitor [1-3] through possible antineoplastic drug and it is given through mouth in the form of tablet dosage. This drug was approved by FDA in September 2019 [4], newly approved by the USA and Chinese drug supervisory establishments for the treatment of (MCL) mantle cell lymphoma [5], liver injury patients [6], and the commercial brand name is Brukinsa, ZBB is used for the action of grown people (adults) with mantle cell lymphoma (MCL) [7-10], who have consumed at minimum one prior treatment [11]. The molecular weight of zanubrutinib ($C_{27}H_{29}N_5O_3$) is 471.56. ZBB was soluble in various organic solvents like ethanol (C_2H_5OH, 5 mg/ml), dimethyl sulfoxide (DMSO, 5 mg/ml), and (DMF, 10 mg/ml) dimethyl form amide, carefully resolvable in aqueous buffers and insoluble in water. ZBB is stored at-20 °C and stable for ≥2 y [12].

Efficiency was assessed in (NCT 02343120) BGB-3111-AU-003, a phase I/II growth, open-label, multi-centre, the single-arm, global trial of B cell malignancies [13, 14], with 32 earlier treated Mantle-cell lymphoma (MCL) patients treated with ZBB managed per day two times with 160 mg [15-17] of ZBB or per day one time 320 mg of ZBB in the form of tablet dosage [18]. Zanubrutinib provides a higher response rate (84%) and prolonged progression-free survival (PFS) in patients with refractory or relapsed Mantle-cell lymphoma (MCL) [19]. These mixtures have established deep replies with several patients achieving undetectable minimal residual disease (uMRD) [20, 21].

Zanubrutinib (ZBB) inhibits (BTK) Bruton's tyrosine kinase by establishing a covalent bond [22] with cysteine 481 remainder in the (ATP) adenosine triphosphate binding abridged of BTK. Adenosine triphosphate binding specificity is generally seen with additional (BTK) Bruton's tyrosine kinase inhibitors. According to the ATP binding profile, zanubrutinib may similarly bind through varying affinities to unrelated and related (ATP) adenosine triphosphate binding kinases that have cysteine residue in this situation. Through blocking the BCR (B cell receptors) signaling pathway, zanubrutinib obstructs the trafficking proliferation, adhesion, and chemotaxis of malignant B cells [13, 14], eventually leading to a decrease in tumour magnitude.

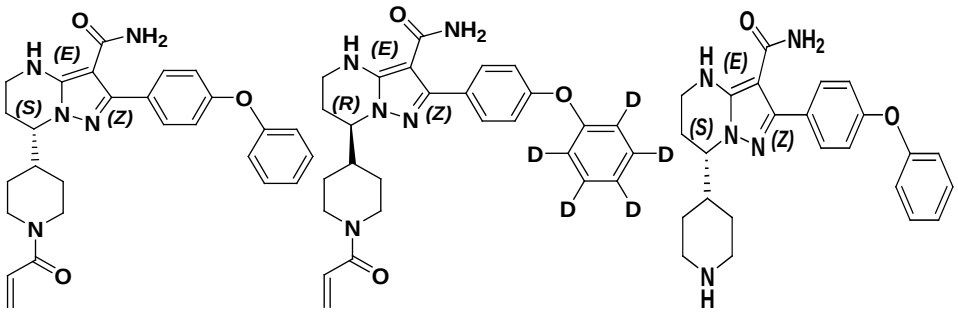
The results of Song, Y *et al.* 2021 [23] give high demonstrate Complete Response (CR) and Overall Response Rates (ORR) in patients with refractory or relapsed Mantle-cell lymphoma (MCL).

A literature survey reported the isocratic analytical technique [24] for the specific determination of zanubrutinib using the RP HPLC technique. The current novel study was reporting technique for the simultaneous estimation of zanubrutinib (fig. 1) and their impurities (table 2) by using the RP-HPLC technique.

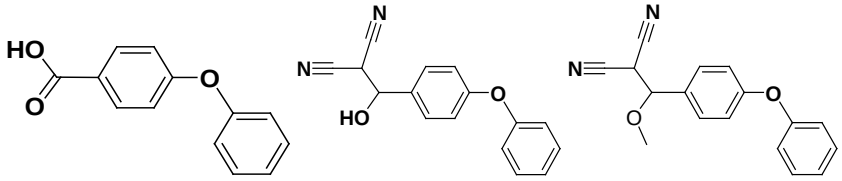
MATERIALS AND METHODS

Waters make instrument is HPLC (Software-Empower 2.0; Alliance model No: e2695) was used for the estimate of the Zanubrutinib and its impurities with the PDA detector. The HPLC grade, methanol, acetonitrile, OPA, Tri fluoro acetic acid, and formic acid used in the mobile phase preparation were purchased from Merck, India. Zanubrutinib is a drug which is present in the form of tablet dosage used in this investigation was purchased from the Mylan laboratory (R and D section), Hyderabad. The Zanubrutinib and their insufficient impurities standards were obtained from Ph. Eur and USP. Reaming impurities were purchased from the Mylan laboratory (R and D section), Hyderabad, India. Unified HPLC systems were

obtained from Waters Corporation, Milford, USA, and prepared by a Waters PDA (photodiode array detector). Empower software 2pro is used for the Information gathering and analysis part. The columns were acquired from Zodiac Life Sciences, HYD, and India. Glassware (Class A) is used in conducting the experimentations and justifications were obtained from Borosil and Duran, India.



a) Zanubrutinib b) Impurity-1 c) Impurity-2



d) Impurity-3 e) Imurity-4 f) Impurity-5

Fig. 1(a-f): Drug class, structural formula, and importance of Zanubrutinib

General procedures

Preparation of standard drug (Zanubrutinib) solutions

Accurately 50 mg of zanubrutinib was weighed and transported to a volumetric (50 ml) flask. Subsequently, proper dissolution with acetonitrile and sonicate for nearly 15 min and a similar solvent was used to dilute up to the mark. The obtained solution utilized the (1000 µg ml^{-1}) stock. Dilute the solution appropriately for additional usage.

Impurity stock solution preparation

Impurity stock solution preparation A

Accurately 5 mg of each of Imp-1, Imp-2, Imp-4, and Imp-5 was weighed and transported to a volumetric (100 ml) flask. Subsequently, proper dissolution with acetonitrile and sonicate for nearly 15 min.

Impurity stock solution preparation B

Accurately 5 mg of each of Imp-3 was weighed and transported to a volumetric (100 ml) flask. Subsequently, proper dissolution with acetonitrile and sonicate for nearly 15 min.

Impurity stock preparation solution

Addition of 1 ml from Impurity stock-A and 1 ml from Impurity stock-B into a volumetric flask (50 ml) and fill up to the mark through the diluents.

Forced degradation and Method validation studies

According to the present rules [28], the last optimized conditions are validated. Diluted stress samples appropriately to prepare the closing concentration holding the planned method conditions (zanubrutinib of 50 µg/ml) and related with blank and standard chromatograms.

Instrumentation

An (HPLC) High-Pressure Liquid Chromatographic method was developed for the estimation of zanubrutinib with impurities; Waters makes instrument is HPLC (Software-Empower 2.0; Alliance model No: e2695) was used for the estimate of the Zanubrutinib and its impurities with the PDA detector with X-Bridge Phenyl (250 mm x 4.6 mm, 5 µm) column, isocratic pump.

RESULTS AND DISCUSSION

Selection of diluent

As per USP general advertisements, the diluent is used to prepare standards, sample solution, impurity standards, placebo solution, and system suitability solutions were nominated based on the solubility studies conducted for zanubrutinib.

Table 1: Solubility study of zanubrutinib at 25 °C

Name of the solvent	Solubility
Water	Insoluble
Methanol	Soluble
Acetonitrile	Soluble
Acetone	Slightly soluble
Ethyl acetate	Slightly soluble

The active compound is easily solvable in acetonitrile and methanol depending on solubility studies. The excipients used in the construction have (USP-NF) combined solubility in weekly acidic pH solution and methanol. Depending on solubility studies (table 1), mobile phase compatibility, and for excipient solubility, A combination of diluent acetonitrile and 0.1% orthophosphoric acid (50:50) was chosen as the diluent for formulating all solutions.

Determination of detection wavelength

In the initial stage of development, recognition wavelength (λ_{max}) was determined by using a UV-Visible spectrophotometer range from 200 nm–400 nm. Then spectra were recorded at the immovable wavelength (λ_{max}) through PDA (Photodiode Array) detector. By using this PDA (Photodiode Array) detector spectral zanubrutinibib and their Impurities were obtained (fig. 2). Zanubrutinib shows four λ_{max}, 225.2 nm, 271.4 nm, 317.8 nm, and 364.2. Table 2 shows the λ_{max} depicted by individual impurities. Based on essential sensitivity for producing the LOQ and LOD for impurities, the suitable UV replies were measured for the Zanubrutinib (225.2 nm).

Wavelength is observed in the present study at 225.2 nm using a PDA detector. Were as Vijayakumari M and Ch Balsekhar Reddy [24] reported 220 nm. But in this study, we use a drug with impurities and advanced software. Hence this method is better.

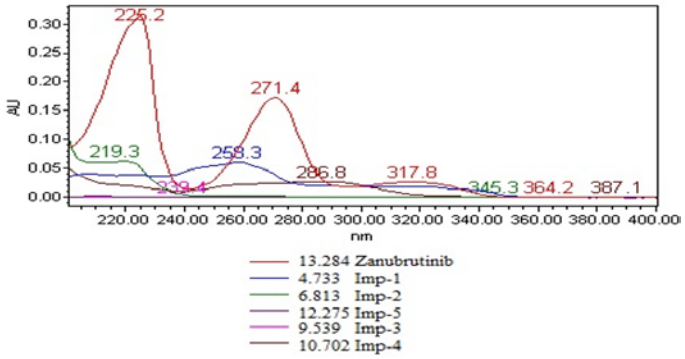


Fig. 2: PDA spectra of zanubrutinib and their impurities

Table 2: Absorption maxima (λ_{max}) of separate impurities

Name	λ_{max} (nm)
Zanubrutinib	225.2, 271.4, 317.8, 364.2,
Impurity-1	207.6, 258.3, 381.0
Impurity-2	219.3, 263.1, 345.3, 383.4
Impurity-3	239.9, 261.9, 288.0, 307.0, 357.3, 391.9
Impurity-4	286.8, 387.1
Impurity-5	208.7, 293.9, 335.7, 353.7, 382.2

Choice of chromatographic situations (Method development)

In the current investigation, to develop technique and validation using Waters make HPLC instrument (Alliance model No: e2695; Software-Empower 2.0). Buffer (0.1% OPA) and acetonitrile (50:50) are used as a diluent for the preparation of normal (standard) solutions of zanubrutinib and their five impurities.

Acetonitrile (LC grade) was selected as the key essential solvent of the mobile phase (MP) because it overdoes the remaining solvents (polar) in the succeeding aspects. (1) Lowermost transmission density (absorbance) at shorter λ max (wavelengths) and hence lesser sound in UV detection. It tells higher sensitivity examination at smaller UV wavelengths (λ max). (2) Less ghost cresting is detected for gradient baselines. (3) Its compatibility is in height with water. (4) it is condensed back pressure and lesser viscosity lead to a considerably greater peak shape. (5) Acetonitrile (CAN) based solutions take higher elution strength related to methanol (CH_3CH_2OH) based solutions. (6) At small mixture ratios, the same retention time can be attained using acetonitrile through smaller than half the ratio of methanol (CH_3CH_2OH). Zanubrutinib compound contains three functional groups: the imidazole ring, amido group, and ester groups. The first one is the imidazole ring, which is a water-soluble, strongly basic, polar nature [25]. The second one is the amido group, which is less basic, less water-soluble, and non-polar in nature. The third one is the ester group, which is less basic, soluble in water up to three carbons but more carbons containing in the compound which is soluble water. Based on the second and third point's compound is insoluble in water [12] and non-polar nature.

According to the principles of RP (reversed-phase), chromatography was used to develop the method. The basis of selecting the RP chromatography is the type and nature of the nominated compounds (Zanubrutinib). For initial expansion trials, the most regular RP (reversed-phase) column, i.e., column C18, with dimensions 150 mm x4.6 mm and element size 3.5µ, was selected (Inertsil ODS). More density bonding of Column C18 (Inertsil ODS) is the most regular RP (reversed-phase) column in which silanols are present in the packing and these are activated in varied pH ranges (pH 2-pH 7.5). Very diluted (TFA) 0.1 %Trifluoroacetic acid is used as a buffer, which acts as an ion association agent in HPLC (liquid chromatography) of compounds like organic [26]. Acetonitrile is used as the medium-polarity solvent [27], which is soluble in non-polar, ionic compounds and it is suitable as a mobile phase inLC–MS and HPLC. Generally, acetonitrile is a favored solvent in (RP-HPLC) chromatography due to its low UV cut-off and lower viscosity.

The initial trial of experimentations was carried out through 60:40, 70:30, 80:20 v/v, Trifluoroacetic acid (pH 2.0), and acetonitrile separately pumped in a mode of isocratic at 1.0 ml min-1 flow rate. During the run time, column temperature was maintained at 25 °C and a 15 min run time was kept. A 10 µl spiked sample (registered impurities) was inserted into the system HPLC (fig. 3). In these trials, no separation of peaks was observed.

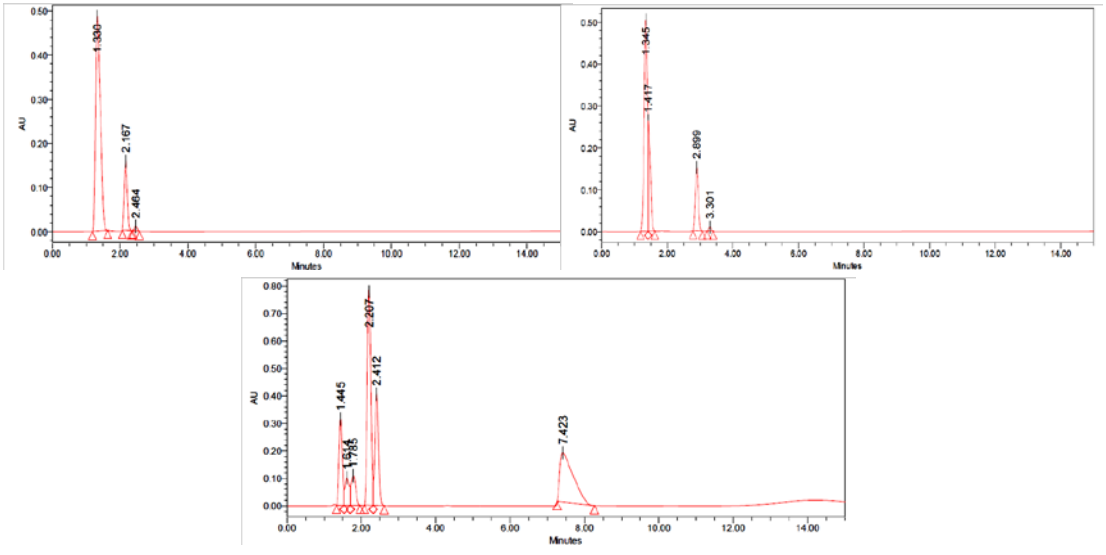


Fig. 3: Chromatograms obtained from column C18 (Inertsil ODS)

Second phase trials were carried out through a change column (Symmetry C18 (150 mm x 4.6 mm, 3.5 µ), change buffer (0.1% formic acid). In these phases, experimentations were carried out through 30+70, 40+60, and 20+80 v/v, 0.1% formic acid (pH 2.7), and acetonitrile separately pumped in a mode of isocratic at 1.0 ml min^{-1} flow rate. During the run time, column temperature was maintained at 25 °C and 15 min run time was kept. A 10 µl spiked sample was inserted into the system HPLC (fig. 4). In these trials, there is the separation of peaks was observed in the first composition, but the plate count was low. The sixth peak is not eluted in the second and third compositions, and base drift is observed.

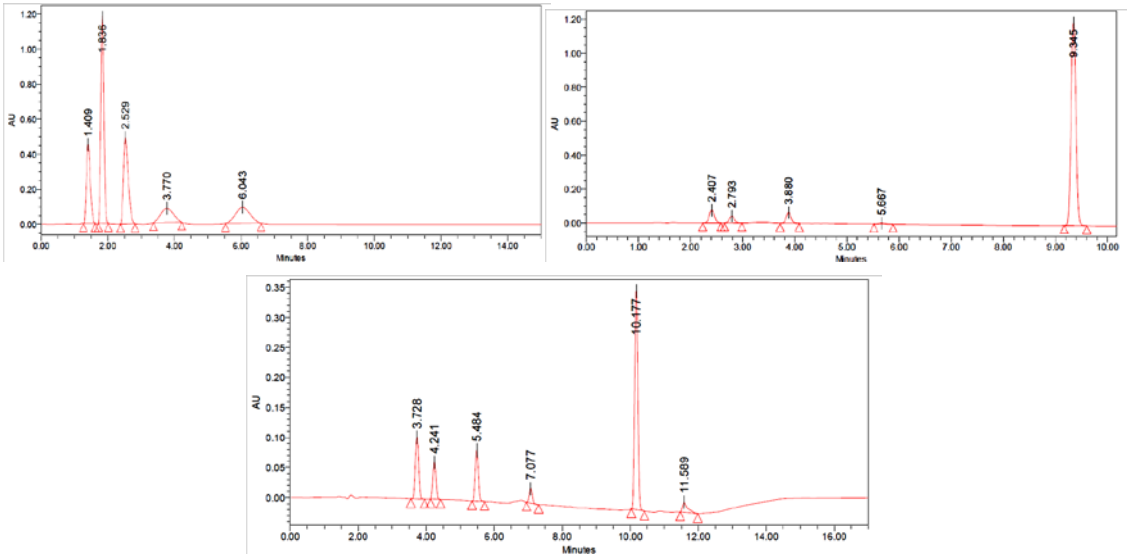


Fig. 4: Chromatograms obtained from symmetry C18 (150 mm 4.6 mm, 3.5 µ)

Further third phase trials were carried out by to change in buffer with 0.1% OPA and same column (Symmetry C18 (150 mm x 4.6 mm, 3.5 µ), In this phase experimentation, were carried out through 20+80 v/v, 0.1% OPA (pH 2.7) and acetonitrile separately pumped in a mode of isocratic at 1.0 ml/min flow rate. During the run time, column temperature was maintained at 25 °C and a 15 min run time was kept. A 10 µl spiked sample (specified impurities) was inserted into the system HPLC (fig. 5). In these trials, less plate count and below two resolutions were observed.

The subsequent fourth phase trial was carried out by changing the column with X-bridge phenyl (150 mm x 4.6 mm, 3.5 µ), 0.1% OPA, Acetonitrile, and methanol. In this phase, experimentations were carried out through 30+50+20, 30+60+10 v/v, 0.1% OPA (pH 2.7), acetonitrile, and methanol separately pumped in a mode of isocratic at 1.0 ml min^{-1} flow rate. During the run time, column temperature was maintained at 25 °C and a 15 min run time was kept. A 10 µl spiked sample (specified impurities) was inserted into the system HPLC (fig. 6). In these trials, the first composition baseline was not proper, and the second composition low resolution was observed.

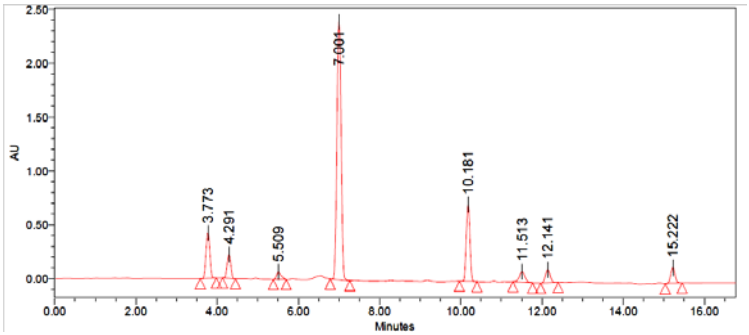


Fig. 5: Chromatograms obtained from (0.1% OPA (pH 2.7) and acetonitrile: 20+80 v/v) as a mobile phase

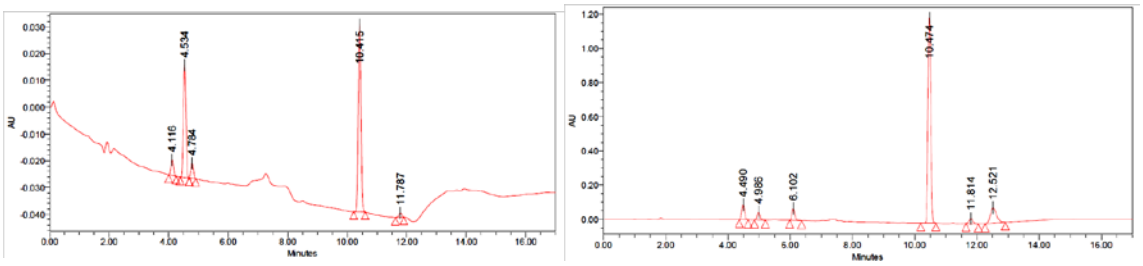


Fig. 6: Chromatograms obtained from X-bridge phenyl (150 mm x 4.6 mm, 3.5 µ)

Finally, the experiment was carried out through 40+40+20 v/v, acetonitrile, 0.1% OPA (pH 2.7), and methanol separately pumped in a mode of isocratic at 1.0 ml min^{-1} flow rate. During the run time, column temperature was maintained at 25 °C and a 15 min run time was kept. A 10 µl spiked sample (specified impurities) was inserted into the system HPLC fig. 7). In this trial was optimized (table 3) because of the good baseline, more USP-plate count, a tail factor is good, and resolution is more than 2.

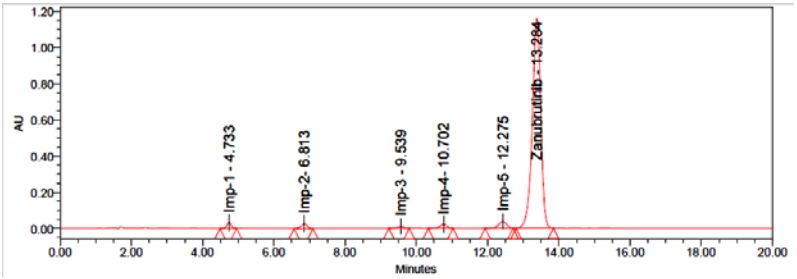


Fig. 7: Chromatograms obtained from (1% orthophosphoric acid: methanol (40:40:20); 40+40+20 v/v/v) as a mobile phase

Table 3: Chromatography conditions of the method

Parameter	Condition
Diluent	Acetonitrile: 1% Ortho Phosphoric acid (50:50)
Detector wavelength	225 nm
Run time	20 min
Column temperature	Ambient
Sample temperature	Ambient
Injection volume	10 µl
Flow rate	1.0 ml min^{-1}
Column	X-Bridge Phenyl (250 mm x 4.6 mm, 5 µm)
Separation Mode	Isocratic
Mobile phase	Acetonitrile: 1% Ortho Phosphoric acid: methanol (40:40:20)

M. Vijaya Kumari and Ch. Balasekhar Reddy [24] reported the mobile phase composition (acetonitrile and 0.1% orthophosphoric acid (50:50 v/v)), theoretical plate counts (6527), and tailing factor (1.04). But the present study reported mobile phase composition (acetonitrile, 0.1% OPA (pH 2.7), and methanol (40:40:20 v/v/v)), theoretical plate counts (15528), resolution (2.17), tailing factor (1.07), Purity1 angle (0.054), Purity1threshold (4.049) and Purity1 flag (No).

Method validation

According to ICH (2005) [28], guidelines (Q2R1) to develop and optimize the HPLC technique were validated. Every validated parameter is discussed in detail in section (table 4).

System suitability and system precision

Optimized method conditions Parameters (system suitability) are in Table 5. The USP tailing factor for zanubrutinib, and its impurities is 1.01-1.09, representing the symmetrical peaks around its axis and take accurate Gaussian shapes. USP plate count (15749-8224) shows that the chosen column effectively resolves the sample components by attaining a good resolution between the neighboring peaks. So, system suitability was verified (USP 40-NF35). In the system precision study, six repeated samples of zanubrutinib were injected and calculated %RSD as 0.138 (<1%), which represents that the made quantities are precise. System suitability and precision chromatograms are shown in Fig. 8 and Fig. 9.

Table 4: Key parameters of validation

S. No.	Parameter	Results observed					
		ZBB	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
1	API Concentration (µg ml^{-1})	50	1	1	0.50	1	1
2	Linearity (µg ml^{-1})	5-75	0.1-1.5	0.1-1.5	0.1-1.5	0.1-1.5	0.1-1.5
3	Method precision (% RSD)	0.417	0.137	0.514	0.741	1.349	1.027
4	Intermediate precision (% RSD)	0.348	0.273	0.597	0.691	0.662	1.041
5	% Recovery	0.117-0.650	0.136-0.417	1.41-0.270	0.652-1.05	1.919-0.340	0.250-0.736
6	LOQ (µg ml^{-1})	0.707383	0.014029	0.052071	0.0365787	0.14673956	0.008431
7	LOD (µg ml^{-1})	0.233437	0.00463	0.017183	0.0120709	0.04842405	0.002782

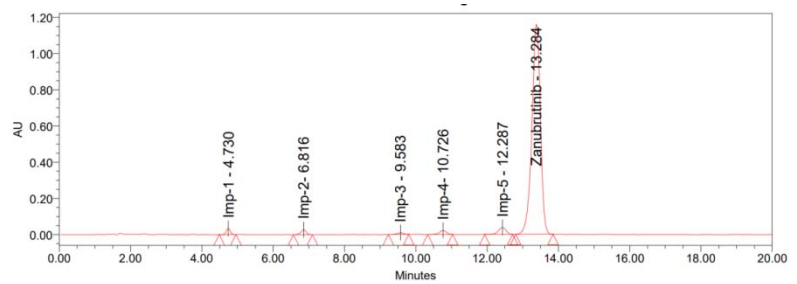


Fig. 8: Typical chromatogram of system suitability

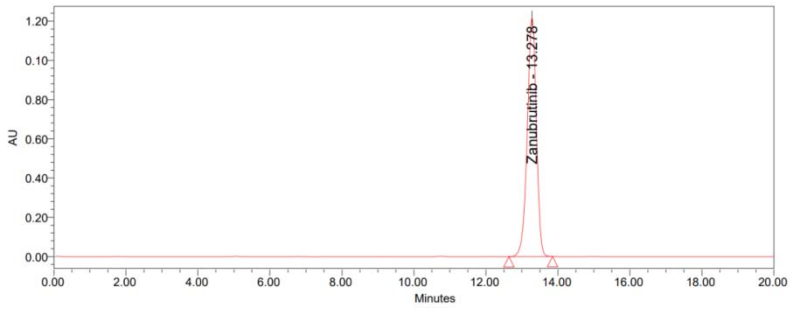


Fig. 9: Typical chromatogram of system precision

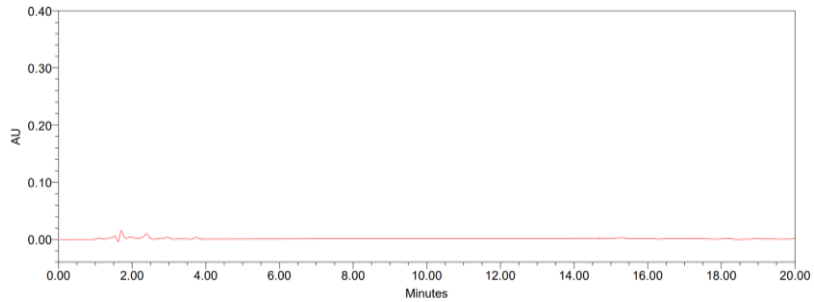


Fig. 10: Typical chromatogram of diluents

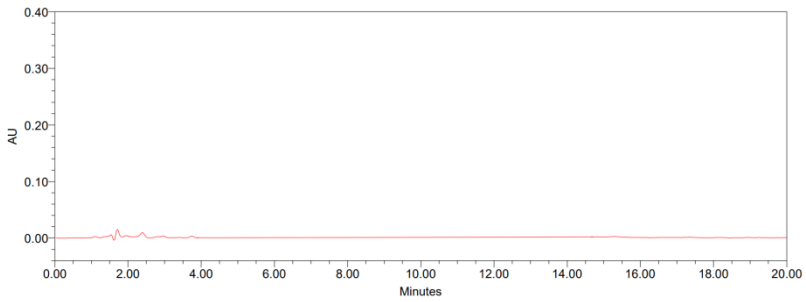


Fig. 11: Typical chromatogram of placebo

Table 5: System suitability parameters at optimized conditions

S. No.	Parameter	Parameter values					
		Zanubrutinib	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
1	Retention time (min)	13.284	4.730	6.816	9.583	10.726	12.287
2	Peak area (µV*Sec)	19194238	263228	280819	119688	274361	577344
3	USP tailing	1.07	1.02	1.05	1.01	1.08	1.09
4	USP plate count	15528	8224	11325	15749	15652	15143
5	USP resolution	2.17	---	8.88	9.64	3.36	4.42
6	Purity1 angle	0.054	0.545	10.543	0.763	0.671	1.127
7	Purity1threshold	4.049	5.278	19.921	6.349	5.420	6.032
8	Purity1 flag	No	No	No	No	No	No

*Purity Flag 'No' indicates the peak is homogenous (Given by Empower software). Peak is Homogeneous if the purity angle is less than the purity threshold.

Specificity

Diluent interference

To prepare the diluent solution as per the improved method, insert it into the chromatographic system (fig. 10). No interference was observed by the retention times of active and impurities peaks owing to the diluent.

Placebo interference

To prepare a Placebo solution as per the improved method, insert it into the chromatographic system (fig. 11). No interference was observed by the retention times of active and impurities peaks owing to the placebo.

Interference from degradation products

The information about the degradation experiment (table 6) given that the high degradation of zanubrutinib was detected in photolytic degradation (UV) (4.64) and the least degradation of zanubrutinib was detected in acid hydrolysis (1.05). Hence ZBB performs less stable in UV rays and highly stable in acidic medium. The technique is precise towards Zanubrutinib and impurities, as the purity threshold is more than the purity angle in entirely stress

parameters, representing the homogeneous analysed peak. M. Vijaya Kumari and Ch. Balasekhar Reddy [24] gives information about the degradation of Zanubrutinib, which was detected in acid hydrolysis.

Table 6: Results of stress study and peak purity data of zanubrutinib

Parameter	Stress conditions	% Assay of the degraded sample (A)	% Degradation w. r. t. control*(B)	Purity angle	Purity threshold
Control sample (No degradation)	No Exposure	98.69159	----	0.054	4.049
Acid hydrolysis	0.1 ml of 1N HCl and heated at 70 °C for 1 h	99.72321	1.04692	0.028	4.030
Base hydrolysis	1 ml of 1N NaOH and heated at 70 °C for 1 h	100.1014	1.43015	0.021	4.050
Oxidation	0.5 ml of 30% H_2O_2 at 70 °C for 1 h	100.0909	1.41954	0.028	4.030
Hydrolysis degradation	Heated at 70 °C for 30 min	100.3068	1.6383	0.026	4.038
Reduction degradation	1 ml of 10% Sodium bisulfate and heated at 70 °C for 1 h	100.0308	1.35862	0.029	4.035
Thermal degradation	Exposed at 80 °C for at least 72 h	100.7019	2.03858	0.052	4.624
Photolytic degradation (UV)	Exposed to 1.2 Million lux hours of light	103.2648	4.63553	0.024	4.037

**B=* (98.69-A)/98.69*100

Linearity

Zanubrutinib (5-75 µg/ml), their quantified impurity-1, impurity-2, impurity-4, impurity-5 (0.1-1.5 µg/ml), and impurity-3 (0.1-1.5 µg/ml) are proved through linearity method in between LOQ to 75 quantified levels (table 7). Various diluted solutions are prepared from standard solutions; these solutions are used we got chromatographed. These chromatograms recorded the response area. By using the linear regression technique, data was estimated. Outcomes of linearity and parameters of regression plotted (table 10). Linear correlation is present between detector response and concentration; hence this technique was linear (fig. 12).

M. Vijaya Kumari and Ch. Balasekhar Reddy [24] information about the linearity of zanubrutinib was carried out in 2-30 µg/ml in bulk and pharmaceutical dosage form but present study, we are using a drug with 5 impurities and advanced software (Empower).

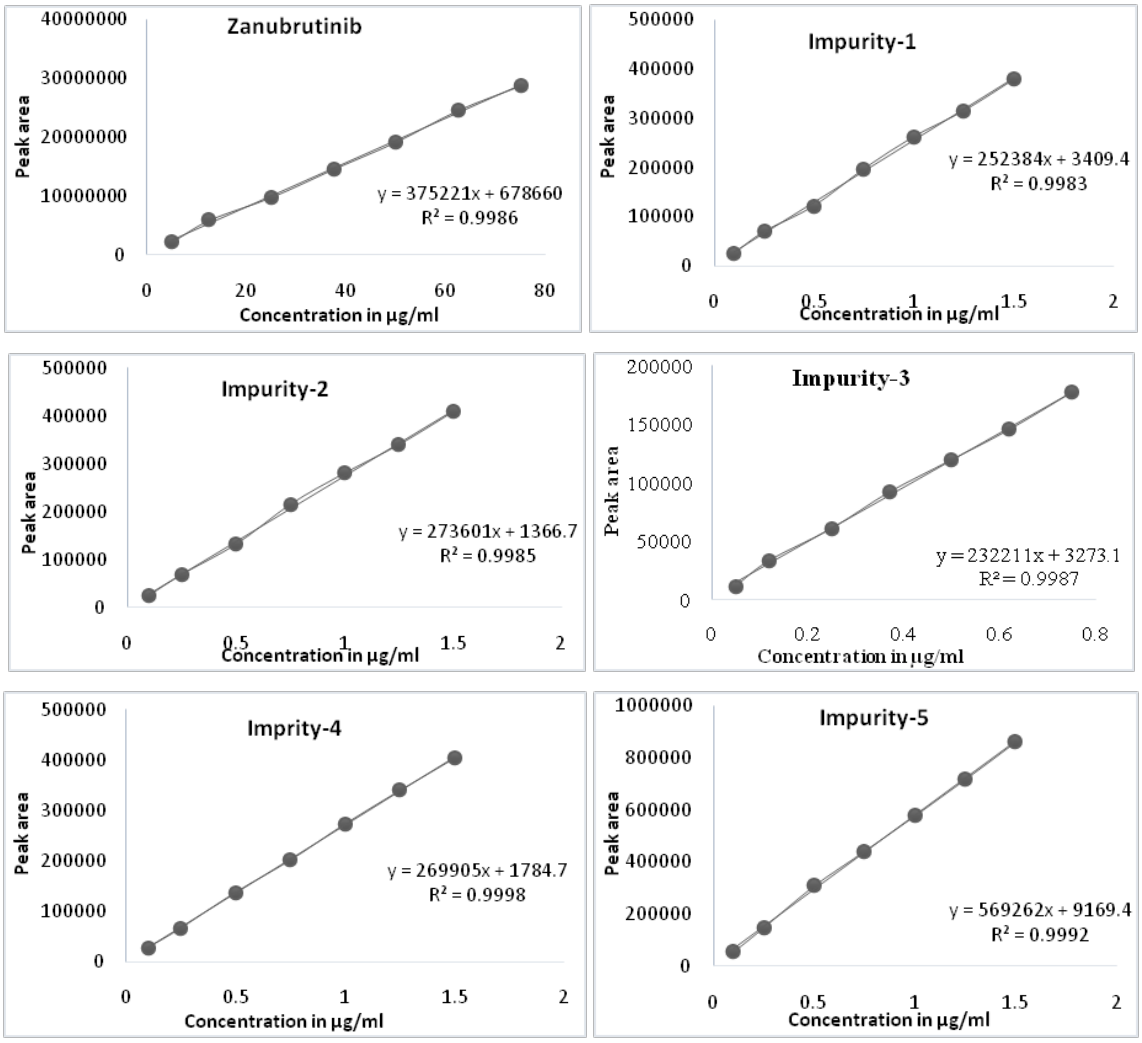


Fig. 12: Linearity graphs of zanubrutinib and their impurities

Table 7: Data from the linearity experiments for zanubrutinib and their impurities

S. No.	Zanubrutinib		Impurity-1		Impurity-2		Impurity-3		Impurity-4		Impurity-5	
	Con	Peak area	Con	Peak area	Con	Peak area	Con	Peak area	Con	Peak area	Con	Peak area
1	5	2244697	0.1	25994	0.1	26336	0.05	11641	0.1	28795	0.1	55162
2	12.5	5971815	0.25	70008	0.25	69804	0.12	33524	0.25	67534	0.25	149494
3	25	9850960	0.5	122200	0.5	132539	0.25	61008	0.5	138619	0.5	308936
4	37.5	14621611	0.75	197825	0.75	215752	0.37	92023	0.75	202290	0.75	441678
5	50	19194238	1	263228	1	280819	0.50	119688	1	274361	1	577344
6	62.5	24559172	1.25	315593	1.25	339824	0.62	145493	1.25	340591	1.25	716875
7	75	28679705	1.5	379273	1.5	408256	0.75	177216	1.5	404295	1.5	860248

Table 8: Summary of regression parameters

S. No.	Parameter	Obtained values					
		ZBB	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5
1	Residual sum of squares	0.9986	0.9983	0.9985	0.9987	0.9998	0.9992
2	Slope	375221	252384	273601	232211	269905	569262
3	Y-Intercept	678660	3409.4	1366.7	3273.1	1784.7	9169.4

Accuracy

The accuracy (table 9) of the investigative method expresses the degree of the nearness of the gained outcomes near the accurate values. A study of the recovery (accurateness) was performed on a recognized quantity of placebo through an increase in the quantified impurities of zanubrutinib at the description level. Models were arranged as per the proposed technique at different levels, i.e., LOQ, 50%, 100%, and 150% of goal analytic concentrations in (n=3) triplicate for the drug and its impurities. Separately percent recovery, (n=3) average percent recovery, standard deviation, and percent relative standard deviation were calculated for the above drug and specified impurities. The separate % recovery was present between 100.18-95.85, 101.71-94.88, 100.20-95.85, 103.15-97.36, 101.23-96.96, 99.18-93.80, which is a good and acceptance range (amongst 85%and 115%) for drug and specified impurities. The percent relative standard deviation values of the drug and specified impurities were calculated between 0.117-0.650, 0.136-0.417, 1.41-0.270, 0.652-1.05, 1.919-0.340, and 0.250-0.736. These outcomes tell that the technique can extract the drug and its impurities after the placebo precisely and accurately (table 9.19-9.22). M. Vijaya Kumari and Ch. Balasekhar Reddy (24) reported accuracy (100.6-99.3) and %RSD 0.33-1.19 of zanubrutinib.

Table 9: Results of accuracy for zanubrutinib/Imp-1/Imp-2/Imp-2/Imp-3/Imp-4/Imp-5

Level of recovery (%)	ZBB		IMP-1		IMP-2		IMP-3		IMP-4		IMP-5	
	Amount recovered (µg ml^{-1})	% Recovered	Amount recovered (µg ml^{-1})	% Recovered	Amount recovered (µg ml^{-1})	% Recovered	Amount recovered (µg ml^{-1})	% Recovered	Amount recovered (µg ml^{-1})	% Recovered	Amount recovered (µg ml^{-1})	% Recovered
LOQ	5.778365	96.31	0.14308	95.38798	0.125249	100.1993	0.056276	0.056276	0.14335	98.8682	0.1886	94.29981
	5.751075	95.85	0.142317	94.87817	0.124577	99.66133	0.057766	0.057766	0.144348	99.55048	0.188283	94.14171
	5.775588	96.26	0.142947	95.29817	0.124741	99.79291	0.056969	0.056969	0.144085	99.36906	0.1876	93.80004
50	24.49124	97.96	0.490826	98.16518	0.493387	98.67749	0.247895	0.247895	0.48904	97.80797	0.493201	98.6402
	24.77454	99.1	0.491095	98.21906	0.482894	96.57881	0.243408	0.243408	0.49303	98.60603	0.490685	98.1371
	24.50794	98.03	0.492958	98.59151	0.482448	96.48963	0.247942	0.247942	0.484809	96.96175	0.49594	99.18793
100	49.32695	99.95	1.031938	101.1704	0.979837	96.06242	0.497663	0.497663	0.990138	98.0335	0.997002	97.74534
	49.35815	100.02	1.030844	101.0632	0.977688	95.85172	0.491682	0.491682	0.988416	97.86293	0.995146	97.5633
	49.44075	100.18	1.024017	100.3939	0.998649	97.90674	0.490252	0.490252	1.022498	101.2374	0.998847	97.92617
150	74.743	99.66	1.524897	101.6598	1.479473	98.63154	0.720797	0.720797	1.469455	97.96368	1.487269	99.15125
	75.01242	100.02	1.525697	101.7132	1.478457	98.5638	0.720375	0.720375	1.473772	98.25143	1.467438	97.82919
	74.76666	99.69	1.521814	101.4543	1.477657	98.51043	0.721761	0.721761	1.50778	100.5187	1.485776	99.05171

Table 10: Comparison of system suitability parameters in precision experiments

System suitability Parameter	Method precision						Intermediate precision					
	ZBB	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5	ZBB	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5
USP resolution	2.423333		8.67	9.388333	3.613333	4.381667	2.518333		8.5	9.406667	3.488333	4.331667
USP tailing factor	1.138333	1.033333	1.048333	1.046667	1.075	1.151667	1.261667	1.075	1.11	1.235	1.268333	1.18
USP plate count	15388.67	8266.5	11429	15565.17	15345	15694.17	15447.83	8252.667	11504.83	15341.67	15581.83	15515.17
Purity1 angle	0.066167	0.551167	10.54933	0.750167	0.658833	1.1595	0.084833	0.55	10.53617	0.740167	0.668333	1.1615
Purity1 threshold	4.0515	5.220167	19.8035	6.342667	5.4485	6.0695	4.0805	5.236167	19.93483	6.348333	5.449167	6.055833
Retention time (min)	13.2675	4.7335	6.824833	9.55	10.58567	12.249	13.29317	4.733	6.818	9.553383	10.51467	12.23317
Peak area	19156813	261719.5	267047.8	110719.2	309280.5	570573	19177458	261910.2	267468.7	112213.3	307824.5	571772.8
SD of area	26542.51	354.0681	1424.665	849.3995	3960.574	479.9309	52211.54	706.8344	1647.169	793.9794	1958.716	479.9309
% RSD of area	0.138554	0.135285	0.511768	0.720527	1.341292	0.84114	0.272255	0.269877	0.593642	0.67165	0.657675	0.84114

* from six standard injections

Values are given in mean±SD; n=6

Table 11: Comparison of method precision and intermediate precision

S. No.	% Assay											
	Zanubrutinib		Impurity 1		Impurity 2		Impurity 3		Impurity 4		Impurity 5	
	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.
1	99.41	99.77	99.26	99.31	99.76	97.95	99.58	99.33	98.96	98.31	98.58	98.58
2	99.74	99.79	99.23	99.19	99.47	98.28	98.51	100.14	97.05	99.96	98.52	98.52
3	100.65	99.99	99.63	99.96	98.17	99.31	98.17	99.27	99.72	99.63	98.76	98.76
4	99.72	99.75	99.42	99.20	99.27	99.32	97.59	98.03	97.06	100.30	98.53	98.53
5	99.70	99.20	99.29	99.37	99.48	99.43	99.64	98.79	100.22	100.12	98.69	98.69
6	100.28	100.37	99.37	99.614	99.43	99.32	98.79	98.41	100.12	99.953	98.64	98.64
Mean (n=6)	99.92	99.81	99.37	99.44	99.26	98.94	98.71	98.99	98.85	99.71	98.62	98.62
Std. Dev	0.416	0.347	0.136	0.272	0.510	0.590	0.731	0.684	1.334	0.660	1.023	1.032
%RSD (n=6)	0.417	0.348	0.137	0.273	0.514	0.597	0.741	0.691	1.349	0.662	1.027	1.041

Values are given in mean±SD; n=6. M. P.: Method Precision I. P.: Intermediate Precision

Method and intermediate precisions

Method precision (M. P.) was estimated by evaluating several (six) samples of a similar batch as per the planned technique. Intermediate (I. P.) precision was estimated by evaluating several (six) samples of a similar batch as per the planned technique on the next day, using different columns and systems. An evaluation of parameters (system suitability) is prepared between the I. P and M. P (table 10) and the % RSD for drug and impurities were less than 1 % which designates that the technique is accurate (table 11). M. Vijaya Kumari and Ch. Balasekhar Reddy [24] reported Method precision (99.7) and %RSD (0.38) of Zanubrutinib.

Solutions stability

Observing the stability methods of [24] sample stability and ordinary solutions is calculated from preliminary stored 24h at room temperature. They have inserted at dissimilar time intervals and modification between preliminary to 24 h % of the assay was not moderately 2%. There is no effect in storage situations for ZBB. The consequences are shown in Table 12.

Table 12: Results of stability of analytical sample solutions (RTand2-8 °C)

Time intervals	Zanubrutinib (% assay)		% Difference	
	RT	2-8 °C	RT	2-8 °C
Initial	104.1552	104.1552	----	------
6h	103.6151	103.5801	0.513566	0.547207
12h	102.8595	102.7252	1.239049	1.367995
18h	102.6992	102.4275	1.392988	1.653825
24h	102.0472	101.9305	2.01903	2.131087

Table 13: Results of LOD and LOQ of zanubrutinib and its specified impurities

	ZBB	IMP-1	IMP-2	IMP-3	IMP-4	IMP-5
LOD	0.233437	0.00463	0.017183	0.012070997	0.04842405	0.002782
LOQ	0.707383	0.014029	0.052071	0.03657878	0.14673956	0.008431

Table 14: Results of the robustness/ruggedness experiment

Altered parameter	Actual cond.	Altered cond.	RT (Min)	Tailing factor	Theor plates	USP resolution	Purity1 threshold	Purity1 angle	peak area (mean±SD)	% RSD
ZBB										
Control	------	------	13.27	1.138	15389	2.42	4.05	0.066	19156813±26542.5	0.139
Flow	1	0.7	16.89	0.587	16574	2.23	4.44	0.235	23623095±25417	0.108
(ml min^{-1})		1.3	11.29	0.827	14551	2.28	4.46	0.267	15546872±91725	0.590
Organic		-5	16.84	1.17	16716	2.70	4.45	0.230	23614479±271510	1.150
solvent (%)		+5	11.39	1.46	14686	2.73	4.44	0.234	16468499±167532	1.018
Wavelength	225	215	13.29	1.16	15384	2.42	4.07	0.246	20278304±36766.5	0.181
		235	13.29	1.14	15456	2.65	4.26	0.152	19528721±143273	0.734
Impurity-1										
Control	---	---	4.73	1.03	8266.5	--	5.22	0.551	261719.5±354.0681	0.135
Flow	1	0.7	5.93	1.08	10523	--	5.56	0.659	217139.3±353.9	0.163
(ml min^{-1})		1.3	3.93	0.98	6543	--	5.55	0.672	321937±517.03	0.161
Organic		-5	5.87	1.16	10487	--	5.56	0.726	326387.7±1124.089	0.344
solvent (%)		+5	3.87	1.32	6650	--	5.65	0.435	224117.7±1680.971	0.750
Wavelength	225	215	4.73	1.10	8247	--	5.26	0.5433	266009±725.1572	0.273
		235	4.73	1.07	8600	--	5.24	0.534	256693±727.5129	0.284
Impurity-2										
Control	------	------	6.82	1.05	11429	8.67	19.80	10.55	278381.2±1424.665	0.512
Flow	1	0.7	8.52	0.976	13561	9.47	19.56	10.76	345733.3±3884.6	1.124
(ml min^{-1})		1.3	5.71	1.06	8133	8.81	19.18	10.76	227593±1652.4	0.726
Organic		-5	8.80	1.22	13142	9.60	19.34	10.73	344999.7±1399.461	0.406
solvent (%)		+5	5.81	1.18	8250	8.60	19.63	10.45	226497.7±1330.494	0.587
Wavelength	225	215	6.82	1.11	11550	8.58	19.92	10.53	265801±1284.874	0.483
		235	6.82	1.14	11501	8.51	19.95	10.56	265508.3±2594.308	0.977

Altered parameter	Actual cond.	Altered cond.	RT (Min)	Tailing factor	Theor plates	USP resolution	Purity1 threshold	Purity1 angle	peak area (mean±SD)	% RSD
Impurity-3										
Control	-----	------	9.55	1.05	15565	9.39	6.34	0.750	117885.8±849.3995	0.721
Flow	1	0.7	11.98	0.937	16495	9.54	6.35	0.748	146509±140.9149	0.096
(ml min^{-1})		1.3	7.98	0.890	13495	8.37	6.37	0.756	95508.67±151.84	0.159
Organic		-5	12.02	0.923	16659	9.48	6.36	0.744	143610±3190.431	2.222
solvent (%)		+5	8.12	1.47	13547	8.49	6.73	0.440	98621±176.0767	0.179
Wavelength	225	215	9.58	1.03	15533	9.69	6.36	0.744	118687.7±208.5793	0.176
		235	9.58	1.21	15656	9.23	6.35	0.764	108471.7±854.8522	0.788
Impurity-4										
Control	------	------	10.59	1.06	15345	3.61	5.45	0.659	295280.5±3960.574	1.341
Flow	1.0	0.7	13.50	0.717	16529	3.65	5.45	0.619	385339±2424.51	0.629
(ml min^{-1})		1.3	9.01	0.613	13752	3.77	5.46	0.638	246729±652.9832	0.265
Organic		-5	13.66	0.683	16602	3.12	5.43	0.669	384864±2184.128	0.568
solvent (%)		+5	9.10	0.653	13447	3.35	5.46	0.649	252017±2267.505	0.900
Wavelength	225	215	10.73	1.08	15514	3.55	5.45	0.650	314673±251.1792	0.080
		235	10.73	1.32	15599	3.46	5.55	0.767	304414.7±2761.849	0.907
Impurity-5										
Control			12.25	1.15	15694	4.38	6.07	1.16	570573±479.9309	0.084
Flow	1.0	0.7	15.72	0.620	16357	4.45	6.16	1.14	725206.3±2912.7	0.402
(ml min^{-1})		1.3	10.40	0.823	15683	4.52	6.21	1.13	466981.3±1530.6	0.328
Organic		-5	15.86	0.470	16435	4.36	6.16	1.16	726737.3±2245.635	0.309
solvent (%)		+5	10.32	0.700	15600	4.58	6.14	1.13	477802.7±631.0882	0.132
Wavelength	225	215	12.29	1.34	15628	4.23	6.12	1.16	578654.3±118.8164	0.021
		235	12.29	1.42	15375	4.61	6.14	1.14	565664±1138.344	0.201

Values are given in mean±SD; n=3

Limits of quantification and detection (LOQ and LOD)

The limit of quantitation (LOQ) and limit of detection (LOD) values were assessed for zanubrutinib, and its specified impurities were tabulated (table 13). These values were calculated from the slope (σ) and standard deviation (SD) method [29-32]. The following formula is used for the calculation of LOD and LOQ [26]. LOD and LOQ values are lower in the present study compared to the other studies [24].

$$LOD = 3\delta * S$$

$$LOQ = 10\delta * S$$

Where, δ = Standard deviation (from peak area)

S = slope of the linearity curve

Robustness

Robustness studies (table 14) were conducted to change the parameters like mobile phase flow rate, organic composition, and wavelength. Changes in flow rate were studied at±3%, organic composition were studied at±5%, and change in flow rate was studied at±10%. Different chromatographic studies like retention time, USP resolution, USP tail factor, plate counts, peak areas, SD, and (n=3) %RSD (table 9.28). This information has a significant effect on the resolution between Zanubrutinib and its impurities. The remaining parameters do not impact the parameters' system suitability. Robustness studies like flow rate and organic changes were reported in M. Vijaya Kumari and Ch. Balasekhar Reddy [24], but the present study reported mobile phase composition, flow rate, organic, and wavelength changes.

CONCLUSION

An innovative RP-HPLC isocratic method was established and then authenticated using Zanubrutinib and its specified impurities (Impurity-1, Impurity-2, Impurity-3, Impurity-4, and Impurity-5). System suitability parameters like USP tailing are good, USP plate count above 4000, and USP resolution is greater than 2 or greater than equal to 2. Obtained peaks are homogeneous because the purity angle is less than the purity threshold and there is No Purity Flag. According to ICH guidelines, this method was validated i.e., all validating parameters like accuracy, precision, and robustness within the limits. Hence this method was chosen for common analysis. Finally, the system-suitable parameters and validation parameters values are within acceptable limits.

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Nil

AUTHORS CONTRIBUTIONS

All authors have contributed equally.

CONFLICT OF INTERESTS

Declared none

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