IJFANS INTERNATIONAL JOURNAL OF FOOD AND NUTRITIONAL SCIENCES
ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

New Bio-analytical LC–MS/MS method development and validation for quantification of Zanubrutinibin human plasma

Govindarao Yedlapalli[1], Y. Ganesh Kumar[2]

[1] Research Scholar, Department of Pharmaceutical Analysis and Quality Assurance, Bir Tikendrajit University, Canchipur, KitnaPanung, Manipur – 795003, India

[2] Research Supervisor, Department of Pharmaceutics,BirTikendrajit University, Canchipur, KitnaPanung, Manipur – 795003, India

***Corresponding author**
Govindarao Yedlapalli
Research Scholar
Department of Pharmaceutical Analysis and Quality Assurance
Bir Tikendrajit University, Canchipur, KitnaPanung,
Manipur – 795003, India
Email: govind057@gmail.com

Article History:
Received: 11-08-2022
Accepted: 25-10-2022
Published: 04-11-2022

Abstract:

The hyphenated methodology that combines spectroscopic and chromatographic techniques is gaining high interest in the pharmaceutical industry. Hence, the objective of present research work was to investigate robust and sensitive LC–MS/MS method for quantification of zanubrutinib in biological samples such as human plasma. The extraction of zanubrutinib from spiked plasma was performed by adopting liquid – liquid extraction with diethyl ether as extracting solvent and dacomitinib as internal standard. The extracted zanubrutinibstandard and internal standard were chromatographed on an Hypersil Gold C18 (50 mm×3.0 mm, 5 µm) columnwith pH 4.2,ammonium formate(5.0 mM) and acetonitrile in 75: 25 (v/v) as solvent A, pH 4.2 ammonium formate(5.0 mM) and methanol in 60: 40 (v/v) as solvent B. Equal volumes of solvent A and B was used as mobile phasepumped at 0.5 mL/minflow in isocratic mode. The analysis was completed within a total chromatographic run time of 4 min that facilitates less time and solvent consumption. The column separated analytes were recorded using mass detector with positive ion electrospray ionization source. The mass spectrum shows precursor-to-product ion transitions at m/z of 472/167 (m+1) for

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

zanubrutiniband 470/321 (m+1) for dacomitinib. The method produces calibration curve linear in 1-500 ng/mL concentration range with sensitive detection limit of 0.30 ng/mL for Zanubrutinib. The mean plasma spiked extraction recoveries of both zanubrutinib and internal standard was very high with acceptable % RSD in all precision studies.The analytes were noticed to be stable in a variety of stability studies performed.The method was validated to be sensitive, accurate and was suitable for determination of zanubrutinibin human plasma and applicable for regular quality analysis studies.

Keywords: Zanubrutinib, LCMS/MS analysis, bio-analytical method, spiked human plasma, stability studies

---

INTRODUCTION:

The hyphenated methodology that combines spectroscopic and chromatographic techniques is gaining high interest in the pharmaceutical industry [1]. In the hyphenated method, the chemical components are isolated from mixtures using chromatography, and the separated compounds are identified using the spectroscopic method [2]. Nowadays, hyphenated methods are frequently employed to address challenging analytical issues. Analytical methods such as UV spectroscopy, HPLC (High-Performance Liquid Chromatography), HPTLC (High-Performance Thin Layer Chromatography) and LC/MS (Liquid Chromatography/Mass spectroscopy) are used for the estimation of drugs regularly [3]. The reliable analytical method known as LC–MShas extremely high sensitivity and specificity. LC–MS–MS is a combination of liquid chromatography (L.C.), which allows for the separation of components, and mass spectrometry (M.S.), which allows the detection, identification, and measurement of component masses even in the presence of other components [4]. The LC–MS/MS is a hyphenated method in which LC and Mass spectroscopy with two mass analysers, whereas LC–MS instruments are essentially HPLC units and Mass spectroscopy with a single mass analyser. The sample components are separated using liquid chromatography (LC), and the divided sample species are sprayed into an ion source at atmospheric pressure, changing them into ions in the gas phase. Ions are sorted using the mass analyser based on their mass-to-charge ratio. The detector may additionally magnify the signal produced by each ion as it emerges from the mass analyser. As a result, a mass spectrum is produced, which can be used to identify the elemental or

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

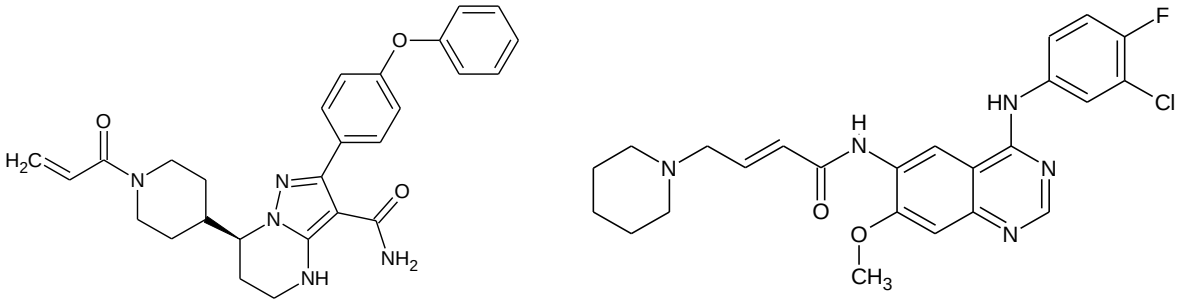
isotopic composition of a sample, as well as the masses of particles and molecules, as well as to clarify the chemical structure of molecules [5].

Analytical method validation guarantees that LC–MS/MS analytical techniques produce reliable and repeatable results. It is an essential step in developing new dosage forms because it provides details about the accuracy, precision, linearity, detection and quantitation limits, and robustness [6]. ICH guideline states that the objective of validating an analytical procedure is to show that it is suitable for its intended purpose. Presently, providing authorities with the validation data is a requirement during the medication development process. ICH and U.S.P. guidelines are among the rules for validating analysis methods [7].

Zanubrutinib (figure 1A) belongs to Bruton's tyrosine kinase (BTK) inhibitor drug and is an anticancer medication prescribed to treat marginal zone lymphoma, mantle cell lymphoma, chronic lymphocytic leukemiaand Waldenström'smacroglobulinemia. Zanubrutinib is classified as a Bruton's tyrosine kinase (BTK) inhibitor [8]. It reduces the tumour size in mantle cell lymphoma by inhibiting proliferation and survival of malignant B cells [9]. The possible side effects associated during the use of zanubrutinib includes diarrhea, constipation, nausea, vomiting, cough, muscle, joint, or back pain, rash, headache and tiredness [10]. It has 471.561 g/mol molecular mass with formula of $C_{27}H_{29}N_5O_3$and its molecular structure was presented in figure 1A.

The literature survey was conducted to determine the methods available for quantification of zanubrutinib. In literature, one analytical HPLC method reported for quantification of zanubrutinib in formulations [11]. One LC-PDA and LC-MS Approach was reported to study the forced degradation behaviour of zanubrutinib [12]. One LCMS/MS method reported for quantification of zanubrutinib in combination with orelabrutinib, ibrutinib and its active metabolite in human plasma [13]. Based on literature review it was identified that there is a need to develop a simple and sensitive bioanalytical method for quantification of zanubrutinib in human plasma. Hence, this study focused to optimize asensitive and stable bioanalytical method for quantification of zanubrutinib in human plasma using dacomitinib (Figure 1B) was utilized as internal standard.

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022



A **B**

Figure1: Structure of zanubrutinib(A) and internal standard dacomitinib(B)

MATERIALS AND METHODS:

Materials:

The zanubrutinibstandard drug with 98.15 % purityand internal standard dacomitinibwith 98.50 % purity were obtained from Sun Pharmaceutical Industries Ltd, Mumbai.The HPLC grade acetonitrile, methanol and milli Q water were procured from Merck chemicals, Mumbai. The local diagnostic laboratory provides the human healthy blood sample.

Equipment:

The analysis was conducted from alliance-2695 (Waters, Japan) HPLC system equipped with triple quadrupole (Waters ZQ, Japan) mass detector, 0. 1 –1500 µL injectable auto-injector and the chromatographic integrations was carried using masslynx 4.2 (Waters, Japan) software. Stuart™ scientific vortex mixer (SA8, Fisher Scientific, USA) was used for vortexing the analytes. The bench top centrifuge (C-852, Remi, India) was used for centrifugation of the analytes. Pasteur pipette was used for separating plasma from whole blood after centrifugation.

Standard solutions:

The zanubrutinib primary stock solutions for preparing standard calibration curve dilutions as well as quality control (QC) samples were prepared using independent weighing's. The zanubrutinib standard stock solution at 1 mg/mLwas prepared using methanol solvent and the prepared stock solution was preserved in a refrigerator at 2-8°C.The working standard dilutions of zanubrutinibwere prepared using appropriate dilutions from standard stock solution using methanol solvent. The dacomitinib working solution at 100 µg/mL concentration was prepared using methanol solvent and then refrigerated.

Calibration curve standards and quality control samples:

The calibration dilutions were arranged by adding 5 mL of selected concentration of analytes to 9.5 mL human plasma. The calibration range consistof eight non-zero concentrations in the

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

level of 1-500 ng/mL of zanubrutiniband 100 ng/mL of dacomitinib. Precision and accuracy samples was set up by spiking appropriate concentration of zanubrutiniband 100 ng/mL of dacomitinib internal standard to blank plasma. The standard zanubrutinibsamples prepared were 1 (LLOQ), 5 (LQC), 100 (MQC), and 500 ng/mL (HQC) and the all the QC levels were spiked with 100 ng/mL of dacomitinib.internal standard. The prepared QC samples were preserved at 6 °C for further use.

Extraction protocol:

In a vortex centrifuge containing 0.5mL of control human plasma, 20 µL of selected concentration of zanubrutiniband 20 µL of internal standard at 100 ng/mLconcentration was added and vortex for one min. Then diethyl ether (4 mL) was added and again vortex for 3 min. The supernatant clear organic layer was separated using Pasteur pipette and dried with nitrogenstream. The dried extract was reconstituted 100 µL using suitable diluent (equal volume of methanol and acetonitrile).

Method development:

The development of method for resolution and estimation of zanubrutinibusing dacomitinibinternal standard was carried by conducting the trails in various method conditions.The method conditions include composition, flow rate, pH of mobile phase, stationary phaseconfiguration and its temperature. The mas operating conditions like collision energy (CE), entrance potential (EP), cell exit potential (CEP) and declustering potential (DP) were optimized. In the process of method development, one parameter change by keeping other in constant and each studied condition, spiked standard solution containing known concentration of zanubrutiniband internal standard was analysed. The results observed in each changed condition was summarized and conditions that produce acceptable results in terms of specificity, symmetry and system suitability was studied for validation.

Method validation:

Bioanalytical method validation guidelines issued by FDA [14] were adopted for validating the method optimized for quantification ofzanubrutinib.

Specificity and Selectivity:

The extraction of analytes was carried for the blank plasma matrix as well as the blank plasma spiked with LLOQ level concentration of zanubrutiniband internal standard. A zero sample that was prepared by spiking internal standard only without analyte was also

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

analysed. The extracted unspiked and spiked plasma samples were analysed and the results achieved were summarized for evaluating method specificity and selectivity.

Linearity and range:

The method was intended to analyse zanubrutinibin human plasma samples and hence the calibration curve dilutions were prepared in human plasma.The anticipated calibration range, peak area response relationship of zanubrutiniband internal standard suggest the number of calibration dilutions prepared for constructing the calibration curve.The calibration curve solutions consists of a blank sample that was spiked with no analytes, zero sample that was prepared by spiking known concentration of internal standard and eight dilutions that covers the anticipated range includes LLOQ.The analysis range in the proposed method was assessed by performing linear regression analysis. Calibration plot was prepared by plotting peak area response ratio of zanubrutinib and internal standard against the prepared zanubrutinib strength.

Accuracy and precision:

Precision and accuracy of the developed method was conducted in six analyses in each concentration and four concentration levels in linearity range. Accuracy was confirmed by evaluating the variation of mean response form the actual value.

The precision experiment was performed as within-run, intra-batch precision that was carried in single analytical run whereas the inter-batch precision or repeatability was conducted in two different analysts in three days.Ruggedness was evaluated by performing the precision by change in analysts. The coefficient of variation in precision study should not exceed 15% in each studied concentration level except LLOQ where in, it should not exceed 20 %.

Recovery:

The recovery of zanubrutinibfrom the spiked plasma need not to be 100 % but the recovery extent of zanubrutiniband internal standard must be reliable, reproducible and precise. Method recovery was performed by correlating results obtained for analyte spiked samples at four (LLQC, LQC, MQC and HQC) concentrations with un-spiked standards.

Matrix effects:

The blank plasma effect on the investigation of zanubrutinib along with internal standard was evaluated in matrix effect. For this, the analyte free plasma of different batches was spiked with LQC and HQC level concentration of both zanubrutinib and internal standard. Then the

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

analytes were extracted and analysed. The area response of zanubrutinib and internal standard was tabulated and the peak area response ratio of zanubrutinib and internal standard was calculated. The % RSD of < 15 % was considered to be having acceptable matrix effect as per the guidelines.

Dilution integrity:

This study was conducted to determine the influence of sample dilution on the precision and accuracy of zanubrutinib in the proposed method. In this, the analytes were extracted from the plasma sample spiked with 5 times higher than HQC level. Then it was diluted to HQC and LQC levels and then analysed. The precision and accuracy was calculated in this study and results under ± 15% was considered as acceptable.

Stability experiments:

The stability of zanubrutinib in the proposed method was determined by conducting different stability studies like freeze–thaw, auto-sampler, dry extract, reinjection, short and long term. All stability studies utilized a set of standards prepared from fresh stock solutions ofzanubrutiniband internal standard in an appropriate analyte and analyte-free plasma matrix. The known concentration of Dacomitinib and internal standard was prepared for evaluating the stability. In all the performed stability studies, the acceptable limit was considered as the % accuracy and % RSD deviation of ±15%.

In short term stability, six aliquots of HQC and LQC concentrations of zanubrutiniband internal standard was preserved for 24 h and then analyzed. The long term stability was performed by storing the HQC and LQC concentrations of zanubrutinibat −20 ± 5 °C and wasanalysed in every three days of analysis. Care should be taken while preparing the sample such that the sample volume must adequate for analysing three separate occasions. The % stability and the % RSD (relative standard deviation) in each time interval were calculated and the long term stability period of zanubrutinibin the developed method was established.

In freeze thaw stability, the HQC and LQC level solution of zanubrutinibwas kept in an air oven at 60 ± 5 °C for 24 h and thawed at room temperature. Then the sample was kept in a refrigerator −20 ± 5 °C for 24 h and then thawed at room temperature. This was considered as one freeze thaw cycle and the process was continued to three cycles. Then the sample was analyzed in the proposed method and results were utilized for evaluating the stability of zanubrutinib.

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

The auto-sampler stability study was performed to evaluate the effect of infrequent delay in sample injection on stability of zanubrutinib. In this, the sample at HQC and LQC level was incubated 24 in auto-sampler and then analysed. The dry reside of analytes after extraction was preserved 24 h at room temperature without reconstitution and then analysed immediately after reconstitution in dry extract stability. Simultaneously a fresh spiked analysis was performed and result obtained in each stability study was compared with fresh spiked analysis results and the % stability was calculated in each study.

RESULTS AND DISCUSSION:

To investigate the quantity of zanubrutinibin plasma samples as well as to evaluate its stability one should adopt suitable method for its quantification.In recent times, rapid and very sensitive analytical technique like LCMS/MS was usually used for bio-analysis of drugs as well as its metabolytes. As the literature doesn't show adequate analytical method for quantification of zanubrutinibin biological samples, this study proposed to develop a simple and sensitive LCMS/MS method to quantify zanubrutinibin biological samples like human plasma.

The mass operating conditions were tuned using spiked plasma solution containing 50 ng/mL of zanubrutinib and 100 ng/mL of dacomitinib internal standard. Intensity of both parent and fragment ion were observed to be significantly prominent in positive mode than negative mode.

The consistent product ion spectra with high intense fragment ions for both zanubrutinib and internal standard were achieved by suitable altering compound parameters like CE, EP, CEP and DP. In addition to the compound parameters, the source dependent parameters such as nozzle voltage, sheath gas flow rate, sheath gas temperature, nebulizer pressure etc., were optimized to achieve satisfactory response of parent and fragment ions. An efficient and fast collision cell which was operated at suitable dwell to enable acquisition with enough data points that produce narrow peaks with nominal sensitivity lose and no cross-talk. Various optimizations were performed and a dwell time was set at 210 ms, at which no cross talk was found. The best detection of mass fragments was noticed at DP of 55 v, EP of 15 v, CE of 40 v and CEP of 20 v.

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

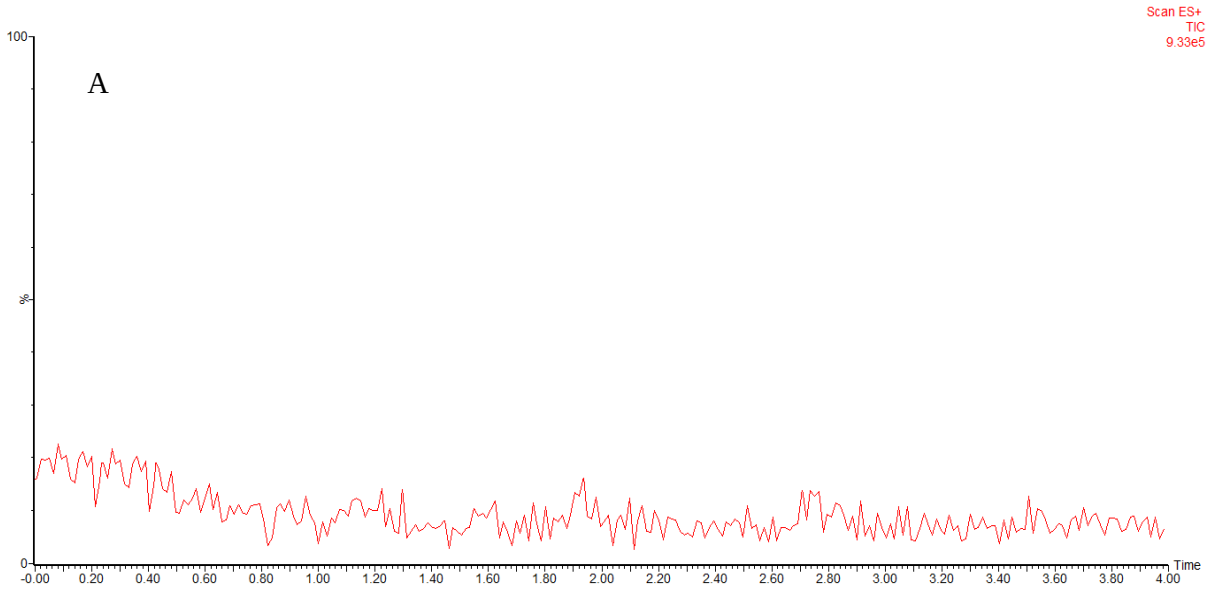
After optimizing mass operating parameters, the chromatographic parameters like column configuration, composition and flow of mobile phase were favourablyoptimized to achieve better quantification results. In the process of method development, methanol and acetonitrile were treated as best organic modifier proposed for LCMS analysis and hence were optimized with different combinations of pH buffers like aceate, phosphate and citrate. Based on the results achieved in each optimization study, the buffer solution containing ammonium formate was confirmed as suitable pH modifier and methanol was finalized as suitable organic solvent for resolving zanubrutinib and internal standard. In the optimization of method development, different configurations of chromatographic columns such as C18 and C8 of different manufacturers was studied to achieve well resolved and retained peaks with short chromatographic run time and free from endogenous components with satisfactory response for both zanubrutinib and internal standard.

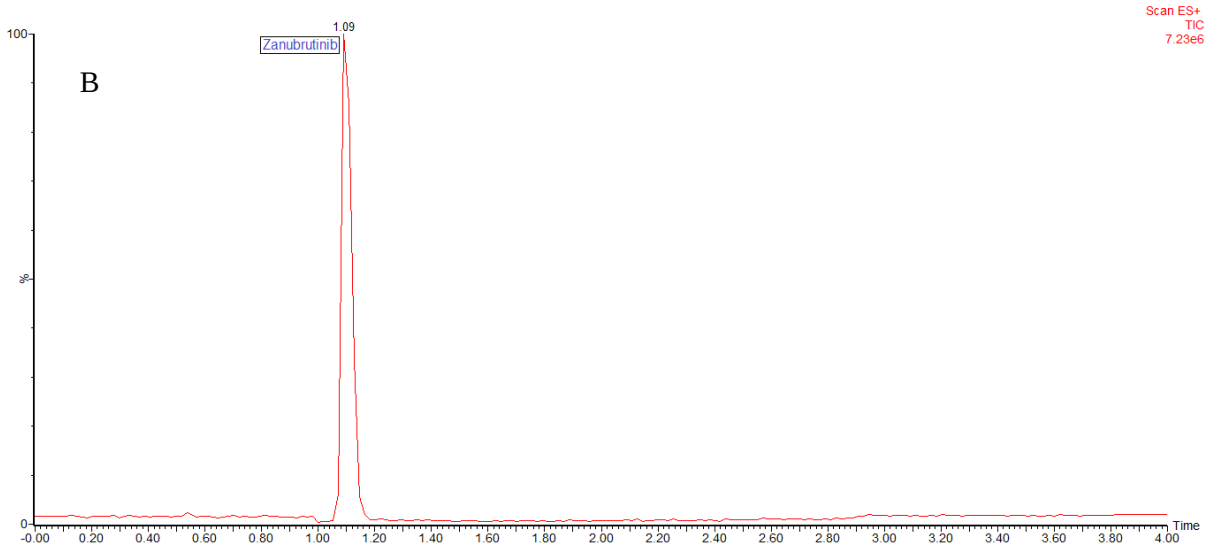
The best chromatographic result was obtained using 5.0 mM ammonium formate pH 4.2 and acetonitrile in 75: 25 (v/v) as solvent A, 5.0 mM ammonium formate pH 4.2 and methanol in 60: 40 (v/v) as solvent B Equal volumes of solvent A and B was used as Mobile phaseunder isocratic conditions. Hypersil Gold C18 (50 mm×3.0 mm, 5 µm) columnresolve the analytes with acceptable symmetry and acceptable peak response LLOQ concentration (1 ng/mL) level. The flow rate of mobile phase was optimized and best resolution of analytes with acceptable peak symmetry was achieved at 0.5 mL/min flow and a shortest 4 min run time to complete the analysis.

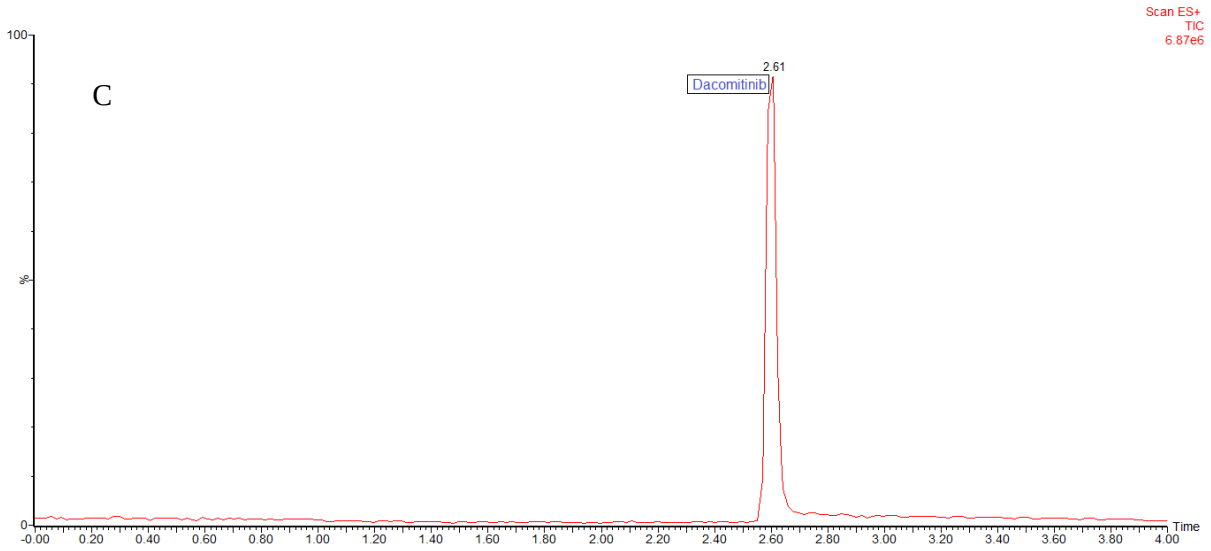
Selectivity and specificity of the optimized method was evaluated by analysing extracts obtained from blank human plasma, combined as well as individually spiked fixed concentrations of zanubrutinib and dacomitinib. As shown in figure 2A, there is no detection of direct interference of plasma traces at the retention time of the zanubrutinib and internal standard. Similarly, figure 2B (plasma spiked with zanubrutinib) and 2C (plasma spiked with internal standard) confirms that no direct interference to the MRM channel of the analyte. The system suitability chromatogram (figure 2D) in the developed method produces symmetric peak at elution time of 1.09 min for zanubrutinib and 2.64 min for Dacomitinib and noticed to be produce satisfactory peak properties as per the guidelines.

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022



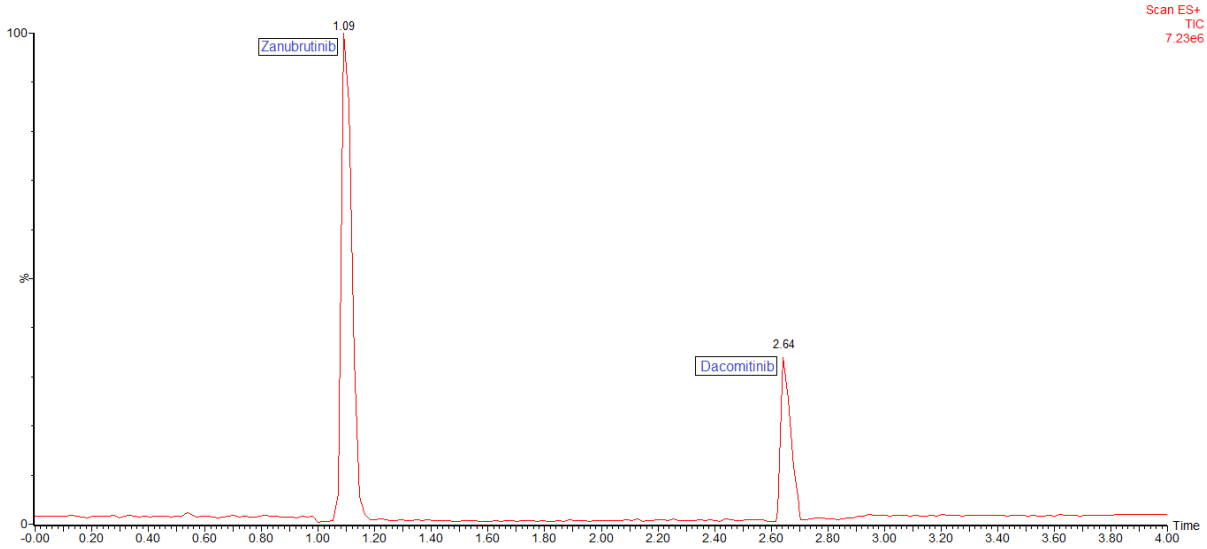




D

ISSN PRINT 2319 1775 Online 2320 7876

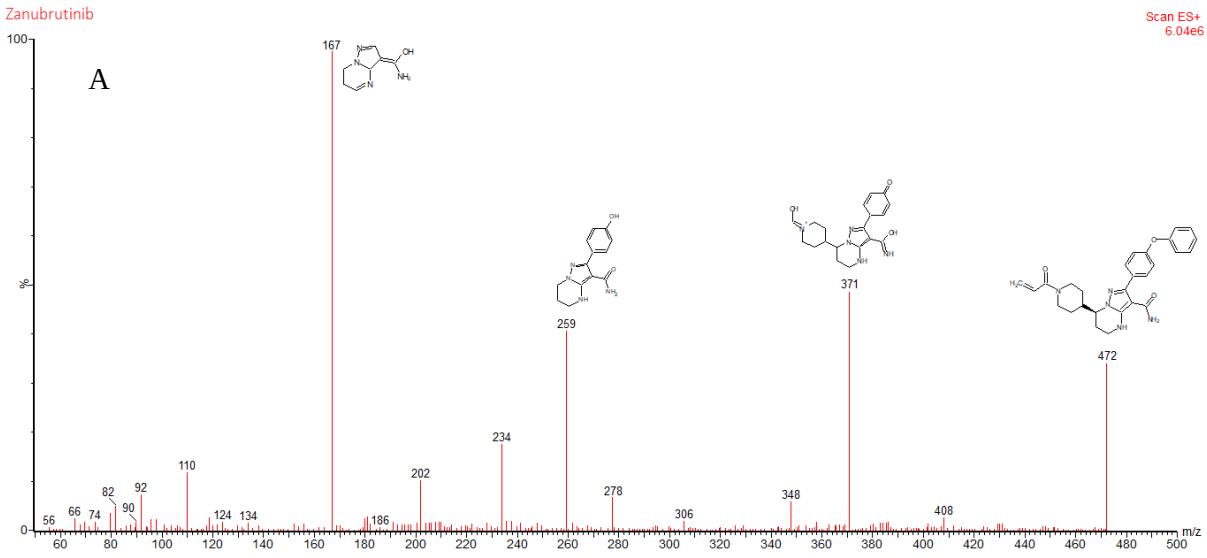
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022



LC chromatogram identified for plasma spiked with no analyte (A); spiked with zanubrutinib (B); spiked with dacomitinib internal standard (C) and spiked with both standard zanubrutinib and internal standard (D)

Figure 2:Chromatogram identified in system suitability study

In the optimized conditions, the Q1 full scan mass spectra shows major protonated $[M+H]^+$ parent ions at m/z of 472 (m+1) and 470 (m+1) for zanubrutinib and internal standard respectively. The most sensitive mass transition was noticed at m/z 167 (m+1) and 321 (m+1) for zanubrutinib and internal standard respectively.



B

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

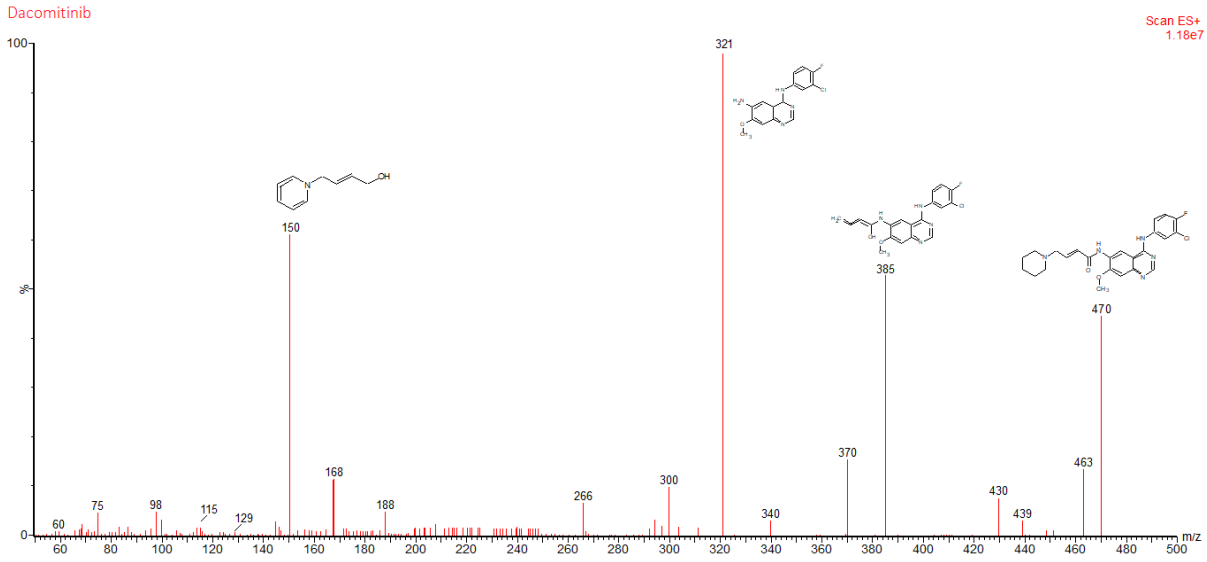


Fig. 3.Mass spectra observed in the developed method showing parent and product ion $[M+H]^+$ of zanubrutinib (A) and internal standard (B)

The reported method employed a convenient, inexpensive and rapid liquid-liquid extraction procedure for extracting analytes form plasma. In the process of extraction, different solvents likeethyl acetate, diethyl ether, tert–butyl methyl ether, dichloromethane and hexane were utilized as solvents for extracting zanubrutinib and internal standard from the plasma. These solvents were tried in individually or combined with each other and in combination with buffers for extracting the analytes. This liquid – liquid extraction avoids the entry of non-volatile compounds on to the column and mass system by producing spectroscopically clean sample and also the cost of the experiment was very low. In the use of solvents other than diethyl ether as extracting solvent produces poor recovery of both standard and internal standard. The clean extracts with high recovery were observed during the use of diethyl ether as extracting solvent. The addition of additives such asformic acid, ammonium formateand ammonium acetate doesn't influence the % recovery of analytes and hence were not used during the extraction process.

The calibration curve was plotted in the concentration level of 1 to 500 ng/mL with y = 0.0077x + 0.0429(R^2 = 0.9998) as regression equation for zanubrutinib. The linearity results presented in table 1 and linear plot presented in figure 1 suggest that the proposed method gives elaborative and sensitive calibration which was adequately suitable for trace level analysis of zanubrutinib.

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

S No	Concentration in ng/mL	Peak area of zanubrutinib	Peak area of internal standard	Peak area ratio ofzanubrutinib and internal standard
1	1	14159.2	343619.5	0.041
2	5	29567.1	344751.2	0.086
3	25	85452.7	342624.3	0.249
4	50	139676.8	341576.9	0.409
5	100	285463.2	342154.8	0.834
6	200	530891.7	343131.5	1.547
7	300	801746.3	342658.9	2.340
8	500	1325691.8	342101.2	3.875

Table 1: Linearity results

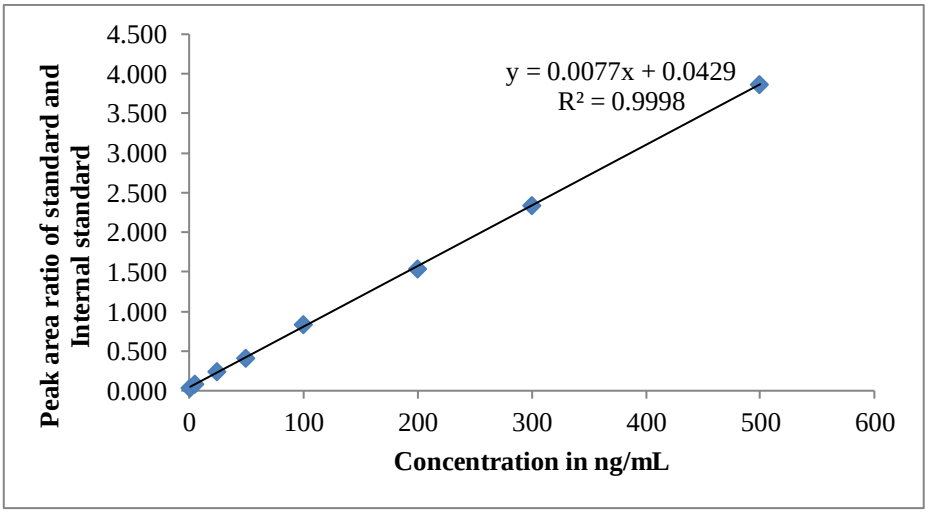


Figure 4: Linear calibration curve

Table 2 presents the summarized results achieved in precision and accuracy study of zanubrutinib in the study. The precision (% RSD) and accuracy values of zanubrutinibwas calculated and was noticed to be within the range of 100.39 ± 1.551 – 97.15 ± 3.080 % in intraday precision, 100.73 ± 1.316 - 97.44± 2.892 % in interday precision and 100.41 ± 1.391 - 98.34 ± 1.689 % in ruggedness study. The % RSD in each studied level was observed to be with in the acceptable level and results revealed acceptable precision and accuracy of the method.

S No	Quality control level	Concentration prepared (ng/mL)	Concentration found (ng/mL)	% accuracy	% Precision (RSD)
Intraday precision (n = 6 at each level)					
1	LLOQ	1	0.97±0.031	97.15 ± 3.080	3.17
2	LQC	5	2.51±0.039	100.39 ± 1.551	1.54
3	MQC	100	98.98±0.753	98.98 ± 0.753	0.76
4	HQC	500	499.53±1.248	99.91 ± 0.250	0.25

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

Interday precision (n = 6 at each level)					
5	LLOQ	1	0.81±0.398	97.44± 2.892	2.97
6	LQC	5	2.51±0.035	100.73 ± 1.316	1.31
7	MQC	100	99.21±0.870	99.21 ± 0.870	0.88
8	HQC	500	498.39±3.081	99.8 ± 0.616	0.62
Ruggedness (n = 6 at each level)					
9	LLOQ	1	0.82±0.402	98.34 ± 1.689	1.72
10	LQC	5	2.51±0.035	100.41 ± 1.391	1.39
11	MQC	100	99.53±0.940	99.53 ± 0.94	0.94
12	HQC	500	497.93±2.039	99.59 ± 0.408	0.41

Table 2-Precision and accuracy results of zanubrutinibin the developed method

The extraction efficiency of zanubrutinib and internal standard in the studied method was evaluated in recovery study by comparing aqueous calibration results. This experiment was conducted in HQC, MQC, LQC and LLOQ levels. The acceptable % recovery of 96.26±0.369, 96.23±0.819, 92.57±0.274 and 92.57±0.274 in HQC, MQC, LQC and LLOQ respectively was noticed for zanubrutinib. Hence it can be confirms that the proposed extraction procedure was adequate for effective extraction of zanubrutinib in plasma samples. Table 3 presents the recovery results of zanubrutinib in this method.

S No	Recovery level	Concentration prepared (ng/mL)	Concentration recovered (ng/mL)	% Recovery	% RSD of recovery
1	HQC	500	481.30±1.845	96.26±0.369	0.38
2	MQC	100	96.23±0.819	96.23±0.819	0.85
3	LQC	5	4.63±0.014	92.57±0.274	0.30
4	LLOQ	1	2.40±0.010	92.57±0.274	0.43

Table 3- Recovery results of zanubrutinib in the developed method

The effect of co-eluting matrix constituents on the extraction and analysis of zanubrutinib in presence of internal standard was evaluated in matrix effect. Matrix effect of both zanubrutinib and internal standard was evaluated in three levels in calibration curve i.e HQC, LQC and MQC. There is no enhancement or suppression results correspond to zanubrutiniband internal standard was noticed suggest that no considerable matrix effect was noticed in the developed method. Hence the proposed extraction procedure and analytical method can effectively remove any probable interfering compounds from plasma matrix.

The dilution integrity study was conducted to determine the influence of sample dilution on precision and accuracy of zanubrutinib and internal standard in the proposed method.Based on the peak area response archived in dilution integrity study, the % recovery and accuracy

ISSN PRINT 2319 1775 Online 2320 7876
Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

was calculated by comparing standard calibration curve. The % accuracy was calculated to be more than 97 % for zanubrutinib in the developed method suggest precise and accuracy of proposed method.

In the process of bioanalytical method development, adequate procedure must be used to preserve the stability of the sample because, instability of the drug produces unreliable data. Hence stability studies need to be performed to determine analyte stability in the developed conditions. A variety of stability experiments namely bench top, freeze–thaw, long term, auto-sampler and reinjection stability had been performed throughout validation.

In all the studied stability experiments, the % stability was calculated to be within ±15% for zanubrutinib in both HQC and LQC levels. The % RSD values in precision study of stability experimnets was calualted to be with in the acceptable limit of less than 15 % (table 4). The results achieved in stability study suggest that the method was stable over the entire validation range.

S No	Stability test	QC level	Amount found (ng/mL)	% stability	% RSD
1	Bench top stability	HQC (500 ng/mL)	494.32 ± 1.498	98.86 ± 0.300	0.30
2		LQC (5 ng/mL)	5.07 ± 0.083	101.45 ± 1.654	1.63
3	Freeze–thaw stability	HQC (500 ng/mL)	195.65 ± 5.385	97.83 ± 2.693	2.75
4		LQC (5 ng/mL)	5.12 ± 0.072	102.56 ± 1.441	1.40
5	Long term stability	HQC (500 ng/mL)	193.60 ± 5.523	96.80 ± 2.761	2.85
6		LQC (5 ng/mL)	5.07 ± 0.063	101.46 ± 1.26	1.24
7	Auto-sampler stability	HQC (500 ng/mL)	193.60 ± 5.523	96.80 ± 2.761	2.85
8		LQC (5 ng/mL)	5.04 ± 0.085	100.90 ± 1.701	1.69
9	Reinjection stability	HQC (500 ng/mL)	190.04 ± 4.668	95.02 ± 2.334	2.46
10		LQC (5 ng/mL)	4.95 ± 0.106	99.06 ± 2.121	2.14

Table 4:Stability studies result of zanubrutinib in the developed method

CONCLUSION:

A simple and convenient HPLC–MS/MS method was proposed and validated according to commonly acceptable US FDA guidelinesfor quantification of zanubrutinib in biological samples like human plasma using similar class drug dacomitinib as internal standard.The reproducible and consistent recovery of zanubrutinib and internal standard was archived with an easy and convenient liquid-liquid extraction using diethyl ether as extracting solvent. The proposed method produces plasma recovered linear calibration curve in concentration level of 1–500 ng/mL for zanubrutinib.The method shows precision and accuracy results in HQC, MQC and LQC levels were within the permissible levels. The stability zanubrutinib in the

proposed method was evaluated by performing extensive stability studies such as bench top, freeze–thaw, long term, auto-sampler and reinjection stability. Acceptable results were produced in the developed method for zanubrutinib in all the stability studies proved the stability of the method. based on achieved results in all method validation studies, it can be confirmed that the method was sufficient for analysis and therapeutic monitoring of zanubrutinib.

REFERENCES:

1. Wilson ID and Brinkman UA, Hyphenation and hypernation: The practice and prospects of multiple hyphenation, Journal of Chromatography A, 2003, 1000(1–2): 325-356 https://doi.org/10.1016/S0021-9673(03)00504-1

2. Coskun O, Separation techniques: chromatography, Northern Clinics of Istanbul, 2016, 3(2):156-160https://doi.org/10.14744/nci.2016.32757

3. ChinazaGodswillAwuchi, HanningtonTwinomuhwezi, Chibueze Gospel Awuchi,Chapter 8 - Hyphenated techniques, Editor(s): ChukwuebukaEgbuna, Kingsley C. Patrick-Iwuanyanwu, Muhammad Ajmal Shah, Jonathan C. Ifemeje, AzharRasul,Analytical Techniques in Biosciences,Academic Press,2022,125-145https://doi.org/10.1016/B978-0-12-822654-4.00015-4

4. Pitt JJ, Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry, ClinBiochem Rev, 2009, 30:19-34

5. Patel K, Patel J, Patel M, Rajput G and Patel H, Introduction to hyphenated techniques and their applications in pharmacy, Pharm Methods, 2010, 1:2https://doi.org/10.4103/2229-4708.72222

6. BhartiMittu AC and Chauhan P, Analytical method development and validation: a concise review, J Anal Bioanal Tech, 2015, 6(1): 1000233

7. International Conference on Harmonization (ICH). Q2A text on validation of analytical procedures; definitions and terminology 60 (1995); USFDA Federal Register.(n.d.)

8. Sawalha Y, Bond DA and Alinari L, Evaluating the Therapeutic Potential of Zanubrutinib in the Treatment of Relapsed/Refractory Mantle Cell Lymphoma: Evidence to Date, OncoTargets and Therapy, 2020, 13: 6573-6581https://doi.org/10.2147%2FOTT.S238832

9. Syed YY, Zanubrutinib: First Approval, Drugs, 2020, 80(1): 91-97https://doi.org/10.1007/s40265-019-01252-4

ISSN PRINT 2319 1775 Online 2320 7876

Research paper UGC CARE Listed (Group -I) Journal Volume 11, Iss 4, 2022

10. HanelW and EpperlaN, Emerging therapies in mantle cell lymphoma, J HematolOncol, 2020, 79: 13 https://doi.org/10.1186/s13045-020-00914-1

11. Kumari MV and Reddy CB, stability indicating validated hplc method for the determination of zanubrutinib in bulk and pharmaceutical dosage form, Asian Journal of Pharmaceutical and Clinical Research, 2020, 13(10): 159-62https://doi.org/10.22159/ajpcr.2020.v13i10.38621

12. Gollu G, Gummadi S, Vemuri VD, Nalla S, Mathala N andAllam S, Forced Degradation Study of Zanubrutinib: An LC-PDA and LC-MS Approach, J ChromatogrSci, 2022, 60(9):807-816https://doi.org/10.1093/chromsci/bmab137

13. Sun LN, Zhao Y, Qian ZY, Chen XL, Ma H, Guo YJ, Shen H and Wang YQ, simultaneous determination of orelabrutinib, zanubrutinib, ibrutinib and its active metabolite in human plasma using LC-MS/MS, Molecules, 2023, 28(3):1205 https://doi.org/10.3390/molecules28031205

14. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV). FDA Guidance for industry: bioanalytical method validation. 2013