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Original Article

BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF IBRUTINIB IN BIOLOGICAL MATRICES BY LC-MS/MS

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ABSTRACT

Objective: The main aim of the research was to develop a fast and highly sensitive bioanalytical LC-MS/MS technique for the quantitation of ibrutinib in human plasma.

Methods: Chromatography has achieved on a reverse phase-symmetry C_{18} (75 mm × 4.6 mm, 3.5 µm) column with gradient elution by acetonitrile, methanol and 0.1%v/v formic acid as the mobile phase. Chromatographic peaks were resolved with 0.7 ml/min flow rate. Drug was extracted with ethyl acetate solvent by liquid-liquid extraction method. Monitoring of transition of m/z 441.2 and 55.01 for ibrutinib and 446.5 and 60.01 for Ibrutinib-D5 were made on multiple reaction monitoring.

Results: Calibration curve of ibrutinib was linear over 1-600 ng/ml concentration range with a regression coefficient (r^2) value of>0.99. The % RSD values were less than 8.5% for inter-day and intra-day precision and accuracy. The method has excellent recovery and the percentage recovery values of lower quality control (LQC), median quality control (MQC) and higher quality control (HQC) samples were 101.86%, 102.8%, and 99.28% respectively.

Conclusion: The drug was stable for more time at variable stability conditions and method was successfully applicable to the regular analysis of ibrutinib in biological matrices.

Keywords: Ibrutinib**,** Chronic lymphocytic leukemia, LC-MS/MS, FDA guidelines and Dilution integrity

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INTRODUCTION

Ibrutinib is a small drug molecule that binds to a protein (Bruton's tyrosine kinase) irreversibly, which is the most important protein in B-cells. It is useful in the treatment of Waldenstrom's macro-globulinemia, lymphocytic leukemia and second-line treatment for marginal zone lymphoma, chronic graft versus host disease and mantle cell lymphoma [1-3]. It is chemically designated as 1-[(3*R*)-3-[4-Amino-3-(4-phenoxyphenyl)-1*H*–pyrazolo [3, 4-*d*] pyrimidin-1-yl] piperidin-1-yl] prop-2-en-1-one. It decreases chronic lymphocytic cancer cell chemotaxis and prevents cellular linkage followed by stimulus at the B-cell receptor (BCR) [4-6]. In addition, ibrutinib down-regulates the expression of CD-20 by pointing the CXCR-4/SDF-1 axis [7]. Ibrutinib inhibits BCR-signaling, which initiates cells into apoptosis or interrupts cell movement and adhesion to protective cancer microenvironments.

In the chronic lymphocytic leukemia (CLL) cell preclinical studies discloses that ibrutinib promotes apoptosis, prevent proliferation and inhibit CLL-cells response to survival stimulus from the microenvironment. This process leads to a decrease of Mcl-1(anti-apoptotic protein) levels in malignant B-cells [7, 8].

Drug literature review discloses that only a few analytical quantification methods for the ibrutinib in bulk, formulations, and biological matrices. The reported analytical techniques were HPLC [9-11] and LC-MS/MS [12, 13]. Goal of the research was to develop a fast and highly sensitive bioanalytical LC-MS/MS technique for the quantitation of ibrutinib in plasma samples and application of regulatory guidelines for method validation.

MATERIALS AND METHODS

Chemicals and reagents

Ibrutinib (Purity: 99.84%) was procured from Hetero drugs PVT. LTD, Hyderabad, India. An internal standard (Ibrutinib-D5) **o**f 99.79% was obtained from MSN Labs, India. Acetonitrile of HPLC grade and formic acid of analytical grade were purchased from J. T. Baker, Hyderabad, India. In the present research work water used from Milli-Q water purification system installed in the lab obtained from Bangalore, India.

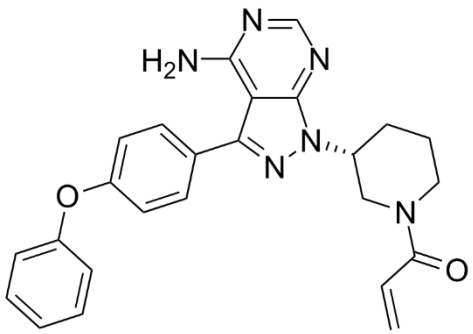

Fig. 1: Structure of Ibrutinib

LC-MS/MS system

A modular Liquid Chromatographic (LC) system (Shimadzu, Japan) equipped with a DGU20-A3 solvent degasser, binary LC20-AD prominence pump,, CTO-ASVP oven for the column and high-throughput SIL-HTC autosampler were utilized for present research.

Chromatography was achieved on a Reverse Phase-Symmetry C_{18} (75 mm×4.6 mm, 3.5 µm) column with gradient elution by acetonitrile, methanol, and 0.1% formic acid as the mobile phase. Chromatographic peaks were resolved by the mobile phase with a flow rate of 0.7 ml/min. Ibrutinib and ibrutinib-D5 internal standard were separated in the total runtime of 6 min. The autosampler temperature and analytical Column temperatures were kept at 5 °C and 35 °C respectively.

The eluents of the liquid chromatographic system were infused into the ElectroSpray Ionization (ESI) source operated with positive ionization method. Starting 0.5 min eluent was avoided from the chromatographic system to evade unnecessary impurities from the various salts existed in the human plasma samples.

In the mass system following conditions were applied; gas-1, nitrogen (40 psi); gas-2, nitrogen (40 psi); temperature of ion source, 400 °C; curtain-gas, nitrogen (25 psi); voltage of ion spray, 5000 V. Monitoring of transition of m/z 441.2 and 55.01 for ibrutinib and 446.5 and 60.01 for ibrutinib-D5 were made on multiple reaction monitoring(MRM). The mass conditions were presented in table 1.

Table 1: Mass conditions for ibrutinib and IS

Compound	Precursor Ion (m/z)	Product Ion (m/z)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Ibrutinib	441.2	55.01	40	8	25	30	5
Ibrutinib-D5	446.5	60.01	100	8	25	82	5

DP: Declustering potential; EP: Entrance potential; CEP: Collision cell entrance potential; CE: Collisional energy; CXP: Collision cell exit potential.

Preparation of standard stock and calibration standards

Stock solutions ibrutinib and IS were processed in 70% methanol at the concentration level of 1000 µg/ml. Quality control(QC) and calibration standard(CC) solutions were processed by spiking blank human plasma sample from the ibrutinib stock solution. CC solutions of eight concentration levels were prepared to produce the final concentrations of 1.0, 5.0, 25.0, 50.0, 100.0, 150, 300 and 600ng/ml. LQC standard, MQC standard, and HQC standards were QC sample solutions and were prepared to produce the concentrations of 2, 200 and 500 ng/ml respectively. All the stock, CC and QC solutions were stored at-20°C till the method of analysis.

Sample preparation

To 250 µl of spiked plasma, 50 µl of Ibrutinib-D5 (1µg/ml) was mixed and sonicated for 15 seconds. To the resulting solution, 500 µl of ethyl acetate was added and vortexed for five min, followed by centrifuged at 5000 rpm for 10-20 min at 5 °C. The organic phase was dried in a lyophiliser. The final residue was dissolved in 200 µl of mobile phase and transferred into a pre-labeled Autosampler vial and infused into an LC-MS/MS system.

Validation

The method of analysis was assessed by validation parameters like sensitivity, precision, linearity, recovery, dilution integrity, accuracy, matrix effect, and stability. Three QC samples of LQC, MQC and HQCs as well as LLOQ (Lower Limit of Quantification) were employed and analyzed in method validation [14, 15].

Specificity and selectivity

Method selectivity was analyzed by comparing the chromatograms obtained from blank and spiked solutions. Method specificity was analyzed by infusing six different lots of blank plasma solutions to ensure no endogenous compounds interfere with ibrutinib and internal standard (IS).

Linearity

CC standards (Non-zero) of 8 different concentrations at 1.0, 5.0, 25.0, 50.0, 100.0, 150, 300 and 600 ng/ml were prepared and analyzed in three separate runs. Linearity curve (peak area ratio of ibrutinib and ibrutinib-D5 peaks against nominal concentration) were plotted by least squares linear regression and reciprocal of the squared concentration ($1/x2$) used as a weighting factor. Deviation should be within±20% for LLOQ and±15% for remaining concentrations.

Precision and accuracy

Intra-day and inter-day precision and accuracy were examined as a part of the precision and accuracy (PA) parameter. Intra-day PA was evaluated by injecting QC solutions (2, 200 and 500 ng/ml) and LLOQ (1.0 ng/ml) in 5 replicates in a day arbitrarily. Inter-day PA was evaluated by injecting the same QC and LLOQ solutions once in a day for 5 different days. The % RSDs for LQC, MQC, and HQCs should be ≤ 20% for LLOQ and ≤ 15% for the remaining QC standards [16].

Recovery and matrix effect (ME)

Ibrutinib recovery was evaluated by paralleling the mean peak areas of extracted and un-extracted samples at low, medium and high QC standard levels. At each concentration level percentage recoveries was calculated and finally, overall mean recovery was calculated. The ME was analyzed by paralleling the un-extracted samples with post-extracted samples [17, 18].

$$\%\ \text{Recovery of ibrutinib} = \frac{\text{Mean ibrutinib peak response in extracted samples}}{\text{Mean ibrutinib peak response in un} - \text{extracted samples}} \times 100$$

Dilution integrity

The sample solution above the upper limit of calibration range was prepared and evaluated for PA parameters. The percentage nominal concentration must be±15 %.

Stability

Stability was analysed at LQC, MQC, and HQC quality control levels. It includes bench-top, freeze-thaw, autosampler and long term stabilities. The bench-top stability was evaluated for 5 h at ambient temperature (25 °C). Freeze and thaw stability was analyzed by storing the QC samples at-70 °C for at least 3 h and for thaw cycle keep the solutions at room temperature. Repeat the freeze and thaw cycles for 3 times. The autosampler stability was analyzed by placing the QC solutions in autosampler at 10-degree centigrade for 8 h. Long term stability was evaluated by placing the QC samples in a freezer at-70 °C for three months [19, 20].

RESULTS AND DISCUSSION

The LC-MS/MS chromatograms of ibrutinib blank, LLOQ, LQC, MQC and HQC concentration levels were shown in fig. 2 and fig. 3.

Method validation

Specificity

From the fig. 2 and 3, system chromatographic conditions were clearly separating ibrutinib and internal standard from endogenous and other plasma substances. The ibrutinib-LLOQ peak response is more than 20% the interference peak response and ibrutinib-D5 peak response is more than 5% from the interference peak response.

Linearity

Ibrutinib calibration graph was linear in the concentration range of 1to 600 ng/ml with the regression equation of Y = 0.4528 X+3.145. The regression coefficient (r^2) value is more than 0.99 which was acceptable as per the FDA regulatory guidelines [14].

Precision and accuracy

Ibrutinib inter-day and intra-day precision and accuracy were analyzed and the % RSD values were calculated for the same and were tabulated in the table 2.

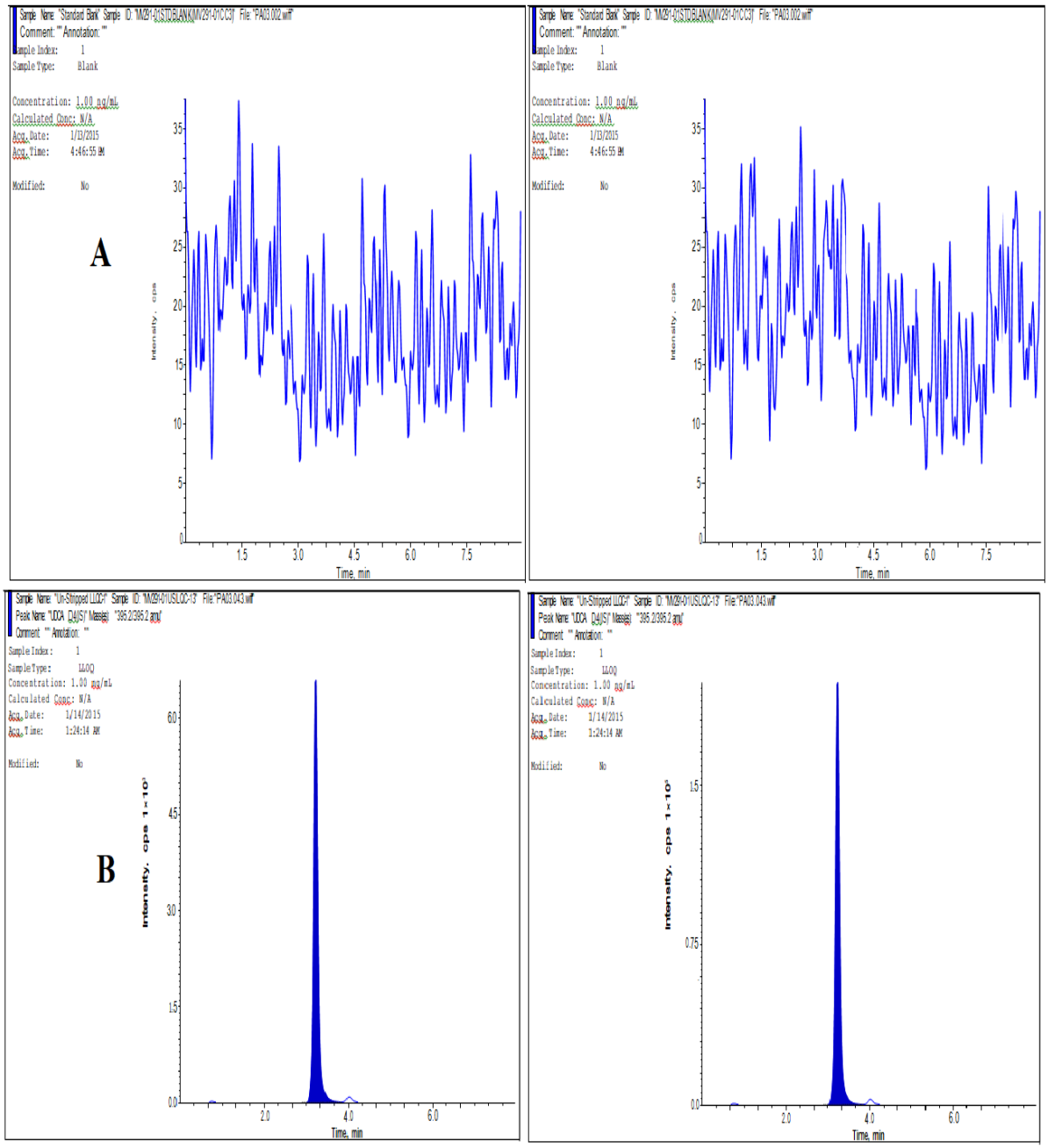

Fig. 2: Chromatograms of (A) blank plasma and (B) spiked LLOQ samples

Table 2: Ibrutinib precision and accuracy data

Nominal concentration (ng/ml)	Intra-day		Inter-day	
	Accuracy (%)	%RSD	Accuracy (%)	%RSD
1	104.6	4.5	107.5	5.2
2	89.6	3.9	91.6	6.1
200	105.2	2.5	101.2	4.2
500	109.8	8.1	96.4	2.9

Recovery and matrix effect (ME)

The method has an excellent recovery and the percentage recovery values were 101.86%, 102.8%, and 99.28% for LQC, MQC and HQC quality control samples respectively. The data for ibrutinib recovery were tabulated in table 3. The matrix effect was evaluated at LQC and HQC level and the calculated %CV values were 4.59% and 3.68% respectively.

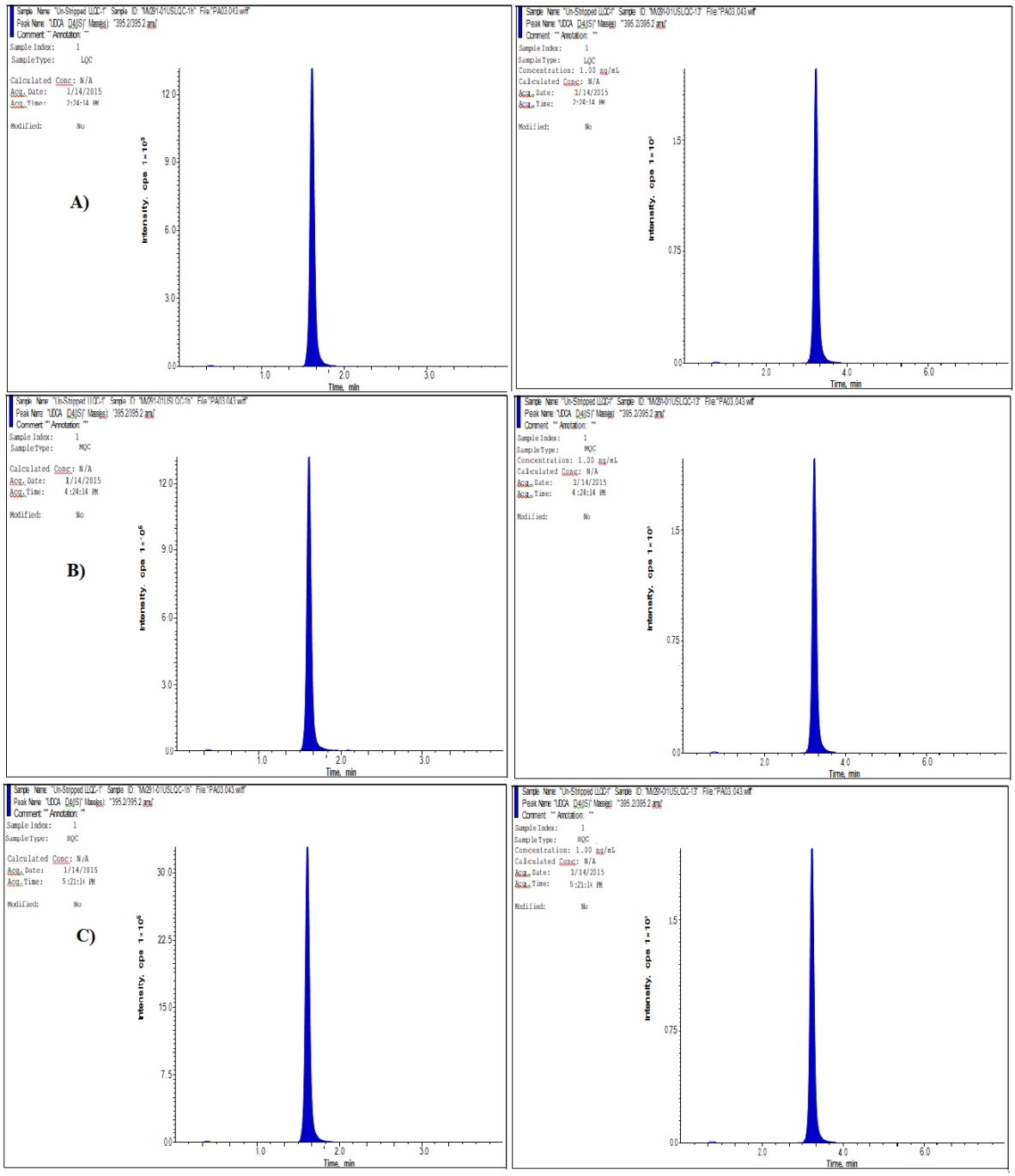

Fig. 3: Chromatograms of ibrutinib spiked (A) LQC, (B) MQC and (C) HQC samples

Table 3: Recovery data for ibrutinib

ID	LQC			MQC			HQC		
	Un extracted (Area ratio)	Extracted (Area ratio)	% Recovery	Un extracted (Area ratio)	Extracted (Area ratio)	% Recovery	Un extracted (Area ratio)	Extracted (Area ratio)	%Recovery
1	0.213	0.251	117.85	0.648	0.634	98.451	0.897	0.885	98.66
2	0.246	0.235	95.59	0.612	0.65	106.20	0.905	0.879	97.12
3	0.254	0.249	98.03	0.598	0.635	106.18	0.856	0.985	115.07
4	0.217	0.215	99.08	0.688	0.643	93.45	0.956	0.912	95.39
5	0.214	0.219	102.34	0.675	0.684	101.33	0.965	0.845	87.56
6	0.243	0.239	98.35	0.598	0.665	111.20	0.895	0.912	101.89
Mean	0.23	0.24	101.86	0.64	0.6525	102.80	0.912	0.903	99.28
SD	0.017	0.014	7.42	0.02	0.017	5.80	0.037	0.04	8.301
%CV	7.29	5.83	7.28	5.68	2.62	5.64	4.11	4.77	8.36

Table 4: Stability for ibrutinib

Drug	Concentration (ng/ml)	Bench-top stability		Autosampler stability		Freeze and thaw stability		Long term stability	
		mean±SD (ng/ml)	%CV	mean±SD (ng/ml)	%CV	mean±SD (ng/ml)	%CV	mean±SD (ng/ml)	%CV
Ibrutinib	2	2±0.4	10.8	2±0.3	6.5	2±0.2	5.2	2±0.2	6.2
	200	200±18.0	9.4	200±11.0	8.9	200±9.0	4.9	200±11.0	5.2
	500	500±21	6.4	500±8.0	4.2	500±7.2	9.5	500±12.0	8.4

Dilution integrity

Dilution integrity of ibrutinib was performed and evaluated. The percentage nominal was within the limit (±15%) and the estimated precision was less than or equals to 15%. It shows that the drug can be dilute to twenty times and the results will be reproducible.

Stability

All the QC standards were exposed to different stability conditions and evaluated to analyse the stability of ibrutinib. From evaluated %CV stability data, the drug was stable for more time at variable conditions like bench-top stability (<10.8%), freeze-thaw stability (<9.5%), autosampler stability (<8.9%) and long term stability (<8.4%) and the values were presented in table 4.

CONCLUSION

A bioanalytical LC-MS/MS method for the ibrutinib was developed and validated with Ibrutinib-D5 as IS. This method has excellent recovery, accuracy, and precision compared with existed methods for the analysis of drug in human plasma samples. The drug was extracted from plasma samples by liquid-liquid extraction (LLE) method with ethyl acetate as an extraction solvent. The drug was eluted within 6 min using Reverse Phase-Symmetry C_{18} (75 mm×4.6 mm, 3.5 µm) column with gradient elution by acetonitrile, methanol and 0.1% formic acid as the mobile phase. The developed technique was validated according to the FDA regulatory guidelines and all the validation parameters were within the acceptable range. The developed technique was effectively applied to the routine analysis of ibrutinib in plasma samples.

AUTHORS CONTRIBUTIONS

All the author have contributed equally

CONFLICT OF INTERESTS

Declare none

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