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Advances in Bioresearch

ORIGINAL ARTICLE

Development and validation of HPLC bioanalytical method for estimation of Ibrutinib in Human plasma

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ABSTRACT
The main aim of the research was to develop a fast and highly sensitive bioanalytical HPLC-UV technique for the quantitation of ibrutinib in human plasma. Celecoxib was used as an Internal Standard (IS) in the development and validation of an HPLC-UV bioanalytical technique for Ibrutinib that is straightforward, sensitive, reliable, and repeatable in human plasma. Methanol, acetonitrile, and 2% formic acid were used in the protein precipitation procedure to make the extraction. Column 18, a polar stationary phase, was combined with a mobile phase. 60:40 v/v acetonitrile: water, 1 ml/min flow rate, and 20 µl injection volume. Internal standard and ibrutinib were retained at 5.77 and 9.83 minutes, respectively.The method was validated over a concentration of six working standard solutions ranging from 1.4 to 56 µg/mL with correlation coefficient 0.999. The run time is about 15 min. 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 93.09%, 94.03%, and 94.95% respectively. The coefficient of variation for intra- and inter-batch testing was ≤15%. A sensitive, selective and robust HPLC method for the determination of Ibrutinib in human Plasma has been developed and validated using celecoxib as an internal standard. In the future, this method can be used for clinical and pharmacokinetic studies.
Keyword: *Bio analytical method, Ibrutinib, Celecoxib, Method Validation, Human plasma*

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INTRODUCTION

Ibrutinib (Imbruvica®, Pharmacyclics, Inc.) is the first irreversible, orally bioavailable inhibitor of Bruton's tyrosine kinase (BTK) and has proven effective in treating several B-cell malignancies. BTK plays an essential role in the tumor microenvironment, which is a complex network consisting of various cells and their precursors. These include pericytes, smooth muscle cells, fibroblasts with diverse phenotypes, myofibroblasts, neutrophils, eosinophils, basophils, mast cells, T-cells, B-cells, natural killer (NK) lymphocytes, as well as antigen-presenting cells such as macrophages and dendritic cells. Collectively, these cells contribute to the development and progression of cancer.

BTK also impacts cell migration and localization, which explains the phenomenon observed with ibrutinib treatment. This involves the movement of lymphocytes from lymph nodes into peripheral blood, resulting in a unique response known as "redistribution lymphocytosis." During this process, lymph nodes shrink rapidly, and the redistributed malignant cells, deprived of survival signals, eventually undergo cell death. On average, this effect resolves within 14 weeks. Similar responses are observed with other inhibitors targeting BTK, SYK, and PI3K pathways. This novel mechanism of action led to the introduction of a new response criterion termed "partial response with lymphocytosis."

Ibrutinib is part of the tyrosine kinase inhibitor class and is specifically developed for the treatment of B-cell malignancies. It was officially approved by the U.S. FDA in 2014, marking a breakthrough in targeted cancer therapies [1-3]. The chemical structure of Ibrutinib was displayed in Fig 1.

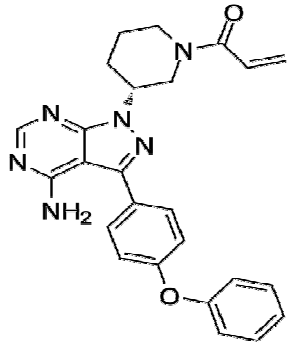

Fig. 1 chemical name: 1-[(3R)-3-[4-amino-3-(4-phenoxy phenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one. molecular weight 440.51 g/mol and formula $C_{25}H_{24}N_6O_2$

Numerous articles discuss the detection of ibrutinib in biological fluids and pharmaceutical formulations; the techniques employed here include ultra-high-performance liquid chromatography coupled to photodiode array detection method [12], liquid chromatographic methods [5,6,7,8,9,10,11], and liquid chromatography-tandem mass spectrometry (LC-MS-MS) [4]. Pharmacopoeia's do not currently have the ibrutinib monograph, and the spectrophotometric method for ibrutinib measurement has not been disclosed.

Determining the design, operating conditions, constraints, and applicability of the method for its intended use, as well as making sure the method is optimal for validation, are the goals of developing bioanalytical methods [9].)

However, a literature review reveals that no method is reported for the determination of Ibrutinib in human plasma by RP-HPLC. Hence, a precise, sensitive, accurate, selective, reproducible, and rapid analytical technique for the estimation of Ibrutinib in human plasma is developed and validated as per ICH guidelines. In the future, this method can be used for clinical and pharmacokinetic studies.

MATERIAL AND METHODS

Fisher Scientific, Thermo Fisher Pvt. Ltd. located in Mumbai, India, provided HPLC-grade acetonitrile (ACN), formic acid and methanol while the Siddhi Lab water purification unit provided HPLC-grade water. Every other reagent used in this study was of analytical quality. Ibrutinib and IS celecoxib was procured from V & S Laboratory, Maharashtra, India.

Equipment:

A UV-visible spectrophotometer (Jasco 550) was implemented using OpenLab EZ Chrome Workstation software to verify the compound sample. HPLC analysis was performed using the Agilent HPLC 1260 Infinity II system equipped with a UV detector. Method development and validation were conducted using OpenLab EZ Chrome Workstation software.

Chromatographic conditions:

First trial containing mobile phase methanol: water 70:30 % v/v gives Ibrutinib eluted at 8.24 minutes with unacceptable chromatography. Guassian peak shape is not observed (Asymmetry = 3.24). Second trial containing mobile phase methanol: water 80:20 % v/v gives Ibrutinib eluted at 4.84minutes with unacceptable chromatography. Tailing observed (Asymmetry = 2.32). Third trial containing methanol: water 90:10 % v/v gives Ibrutinib eluted at 2.86 minutes with unacceptable chromatography. Splitted peak observbed (Asymmetry = 2.10 and Theoretical plates = 1265). Fourth trial containing Acetonitrile: water 70:30 % v/v gives better peak, good retention time, tailing factor therefore chromatographic conditions in trial four was used. Representative chromatograms of trial 1 to 4 are represented in Fig. 2,3,4 & 5

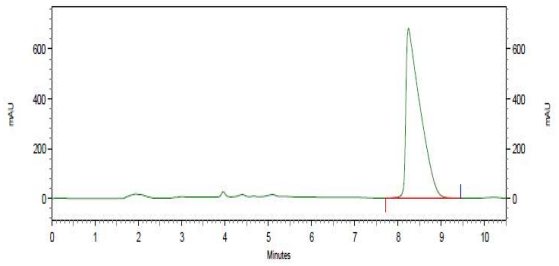

Fig. 2 Trial 1

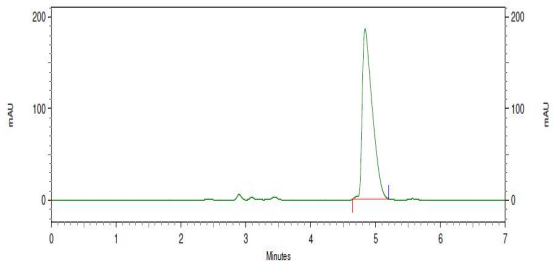

Fig.3 Trial 2

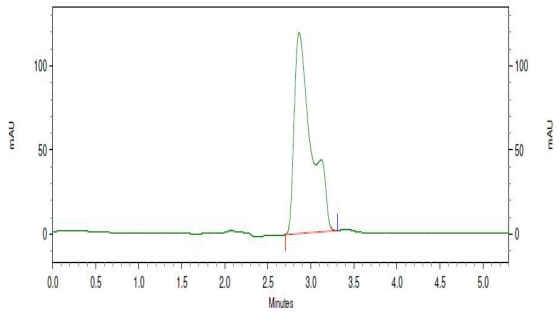

Fig.4 Trial 3

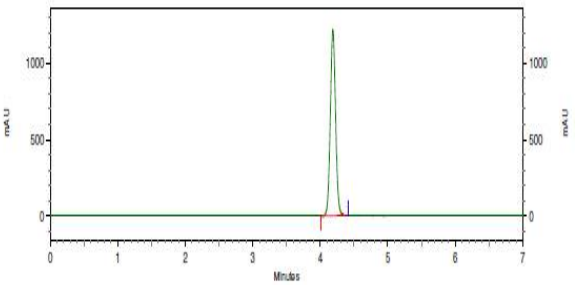

Fig. 5 Trial 4

Using an HPLC method and a Phenomenex Luna omega 5 µm polar C18 column (250 mm X 4.6mm ID) kept at 40ºC, the samples were evaluated. after several trials with different mobile phase including methanol: water 70:30 %, 80:20 % and 90:10 % (v/v) trial 4 using a 40:60% (v/v) ratio of acetonitrile to water, an injection volume of 20 µl, and a total flow rate of 1.00 ml/min, an isocratic condition was maintained during the separation process. A wavelength of 260 nm was used to evaluate the samples. To provide a lasting baseline, the HPLC system was stabilized for 60 minutes at the ideal procedure settings before the analysis was conducted. Ibrutinib was retained at 5.7 ± 0.03 minutes and the IS at 9.8 ± 0.02 minutes for each sample, which needed a total duration of 15 minutes.

Sample preparation process and extraction of Ibrutinib from plasma:

Extraction of Ibrutinib from plasma was done by single-step protein precipitation using methanol containing 2% v/v formic acid and acetonitrile as the precipitant. The biomatrix-based calibration curve (CC) as well as quality control (QC) standards were prepared from working standards (10× concentration) (1.4, 4.2, 28, 45 and 56 µg/mL for CC standards; 1.25, 26.3, and 37.5 µg/mL for IS). A volume of 25 µL each of Ibrutinib and 50 µL IS working standards were externally spiked to 475 µL of thawed blank plasma to make a total volume of 550 µL and mixed. Centrifuged at 5000 RPM for 3 minutes. The 0.5 mL supernatant was collected, placed in a sample loading vial, and injected into the column.

Ibrutinib HPLC method development:

Blank:

No interference at Retention time of Ibrutinib and Internal standard Celecoxib was observed in blank plasma. The blank chromatogram was given as follows in Figure 6.

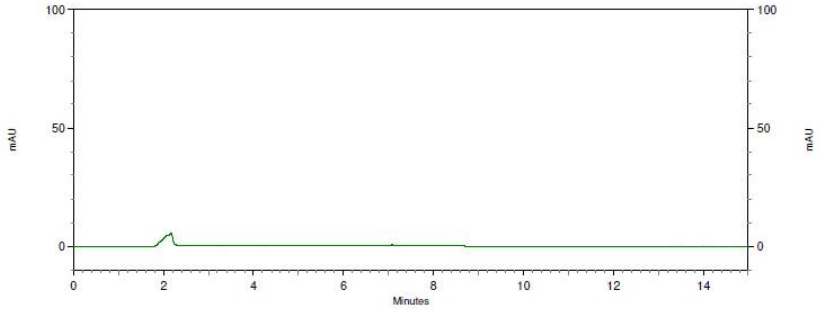

Figure 6: Blank chromatogram

The HPLC method was developed for Ibrutinib with the chromatographic condition as mentioned in Table 1. We found Ibrutinib was eluted at 5.77 minutes with acceptable chromatography as shown in figure 7.

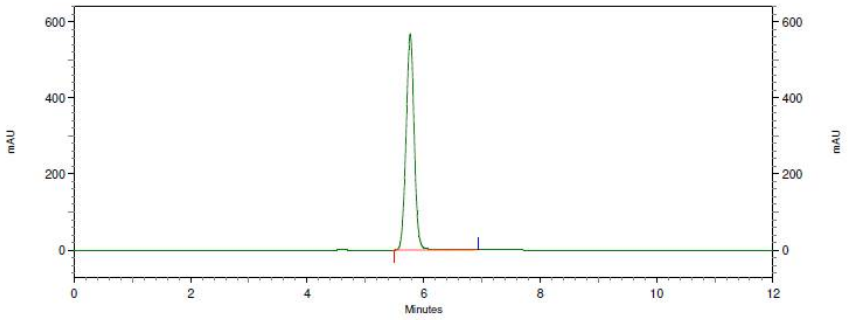

Figure 7: Ibrutinib eluted at 5.77 minutes with acceptable chromatography (Asymmetry = 1.02 and Theoretical plates 8112)

Internal standard HPLC:

Celecoxib was used as internal standard for the current method development. Celecoxib weighed 10 mg of Celecoxib drug and dissolved in 10 mL of methanol (1000 PPM). Pipette out 1 mL of drug stock solution and diluted to 10 mL with mobile phase of optimized trial. Celecoxib was eluted at 9.89 minutes with good chromatography. As shown in Figure 8.

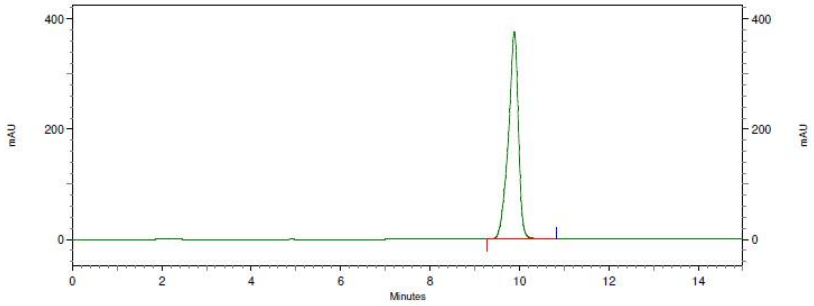

Figure 8: Celecoxib eluted at 9.89 minutes with good chromatography.

HPLC Chromatogram of mixture of Ibrutinib and Celecoxib:

Mixture of 100 PPM each of Ibrutinib and Celecoxib as IS will be injected to check chromatography in mixture. Each stock solution prepared in methanol and final dilution prepared in mobile phase. Each drug shows good chromatography Ibrutinib shows retention time 5.77 min with Asymmetry of 1.02 and 8755 Theoretical plates. While Internal standard Celecoxib shows retention time 9.83 min with Asymmetry of 0.94, 8873Theoretical plates and Resolution 12.25. The chromatogram is represented in Fig. 9

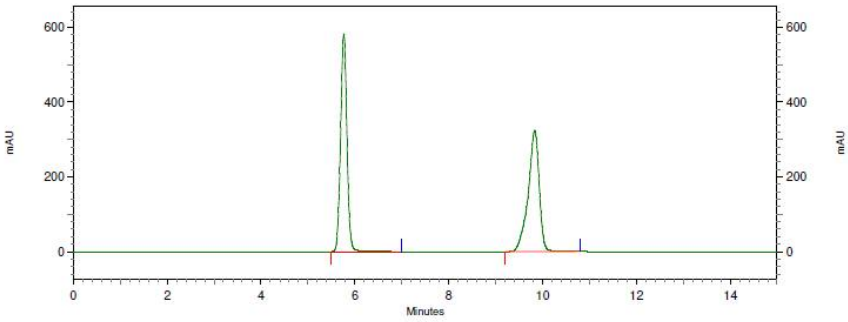

Figure 9: Chromogram of mixture of Ibrutinib and Celecoxib.

Preparation of Internal Standard solution:

475 µL of human plasma + 25 µL of 1000 PPM of Ibrutinib in methanol solution, vortexed for 1 minutes + 50 µL of 1000 PPM of Celecoxib in methanol solution, vortexed for 1 minutes. Added 20 µL of 2 % Formic acid vortexed for 1 minutes. Added 1 mL of Acetonitrile, vortexed for 2 minutes. Centrifuged at 5000 RPM for 3 minutes. Withdraw 0.5 mL of supernatant and injected.

Method Validation:

Preparation of reference standard:

According to USFDA guidelines, reference standards consist of the calibration and QC standards (CCs and QCs, respectively) [13,14]. Ibrutinib's principal stock solution (2000 µg/ml) was made using methanol acting as a diluent. Different working standard solutions (10× concentration) were made from the stock solution for CC and QC standards, as well as IS (28–1120 µg/mL for CC standards, and 28, 84, 560, and 900 µg/mL for QC standards; and 307 µg/mL for IS). Three QC standards—lower QC (LQC, 4.20 µg/mL), medium QC (MQC, 28 µg/mL), and high QC (HQC, 45 µg/mL)—were made from the corresponding working standard solutions, whereas six non-zero CC standards were made in the range of 1.40–56 µg/mL. IS was added to each CC and QC sample at a concentration of 307 µg/mL.

Selectivity:

In order to verify that the sample being tested is the target analyte and to analyze, reduce, or avoid any interferences, the USFDA and ICH guidelines propose evaluating a developed method's selectivity. To establish the selectivity and specificity of the novel method, plasma samples from six different animal sources were analyzed with and without Ibrutinib and IS. For exogenous selectivity, samples were externally spiked with quantities of commonly used excipients in formulations; for endogenous selectivity, 5% v/v hemolyzed material was used to spike the blank matrix.

Linearity and range analysis:

The linearity of the procedure was established using six freshly prepared, non-zero CC standards (1.40–56 µg/mL). The data from eight duplicate (n = 8) CC series were subjected to a least-squares simple linear regression analysis in order to find the mean calibration equation. ANOVA was used to determine the statistical significance of the simple least-square linear regression equation, and the standard error of estimate (SEE) and R 2 adjusted and R 2 forecasted values were used to evaluate the equation's predictability. Additionally, the accuracy (% bias) and precision (%RSD) of the back-calculated concentrations of the CC standards were reported. Ibrutinib concentrations in each trial sample were calculated using the mean calibration equation.

Sensitivity:

1.40 µg/mL is the method's lower limit of quantification (LLOQ). However, using the following equations, as stated in ICH Q2(R2), the mean slope and standard deviation of the Y-intercepts of eight-replicate CC data were also used to determine the limit of detection (LOD), also known as the detection limit (as specified by the ICH Q2(R2)), and the limit of quantification (LOQ), which represent the true sensitivity of an analytical method.

$$LOD = 3.3 \times \frac{[\text{standard deviation } of\ Y - \text{intercepts}]}{\text{average of slopes}} \quad \text{--------- (1)}$$

$$LOQ = 10 \times \frac{[\text{standard deviation } of\ Y - \text{intercepts}]}{\text{average of slopes}} \quad \text{---------- (2)}$$

Accuracy, Precision, and % Recovery:

The percent difference (% bias) between the observed concentration and the nominal concentration for each of the QC standard (n = 18) and CC standard (n = 6) levels was used to calculate accuracy. Three distinct methods were used to determine the method's precision or degree of reproducibility: intra-day (n = 3 samples across all QC levels analyzed twice a day); inter-day (n = 18 samples across all QC levels over 3 different days); and overall (using n = 6 both CC and QC standards at all levels). The relative standard deviation across all replicates is used to express precision.

By comparing the peak area ratios of Ibrutinib/IS obtained from extracted samples from plasma to those of the analytical (aqueous) standards at the same concentration levels, the recovery of Ibrutinib was evaluated for all CC standards (n = 4) and all three QC levels (n = 6) in order to assess the effectiveness and reproducibility of the extraction method.

Storage stability:

In three distinct storage settings—benchtop (25 ± 2 ◦ C), autosampler (15 ◦ C), which most likely resembles the conditions during in vivo PK study sample analysis—the stability of the Ibrutinib-spiked plasma samples over all QC levels (n = 3 at each level) was assessed. Over the course of 24 hours, the bench top stability of the processed QC standards was assessed every 6 hours, whereas over the course of 48 hours, the autosampler stability of the processed QC standards was examined every 24 hours. Additionally, QC standard replicates (n = 3) at all levels were assessed for three cycles of freezing (−20 ◦ C) and thawing (25 ◦ C), in which samples are frozen for at least 12 hours and then thawed.

Ibrutinib's stability was also assessed in a complete blood sample that was kept at -20°C for ten days. The stability analysis of the Ibrutinib stock solution in methanol was also examined over a 30-day period in accordance with the standards. All of the samples underwent stability assessments against the freshly prepared samples in accordance with US FDA and ICH requirements, and the results were expressed as a percentage variation.

Results:

Selectivity:

No interfering peaks were found when chromatograms from the standard and sample solutions were compared in the vicinity of the ibrutinib peak to assess the chromatographic method's selectivity. No interference bands were found when the spectra from the standard and sample solutions were examined in order to assess the spectrophotometric method's selectivity.

Calibration curve for linearity:

The least-squares approach and linear regression analysis were used to prove the methods' linearity. The range of concentrations was 1.4-56 µg/mL. The results of regression analysis showed a high correlation coefficient of 0.999. Table 1 displays the results and Figure 10 represents calibration curve. The RSD% value for each point (n = 3) was less than 2%.

Table 1: Calibration Curve summary

Standards	Actual Conc of Ibrutinib (µg/mL)	Area	Area of Ibrutinib	Avg Area of Ibrutinib	Area	Area of IS	Avg Area of IS	Area Ratio of Anlyte to IS (area of Ibrutinib / Area of IS)	Recovered conc. of Ibrutinib (µg/mL)	% Accuracy
Blank	0	Area 1	ND	ND	Area 1	0	ND	NA	NA	NA
	0	Area 2	ND		Area 2	0				
	0	Area 3	ND		Area 3	0				
Blank + IS	0	Area 1	ND	ND	Area 1	9753029	9754009	NA	NA	NA
	0	Area 2	ND		Area 2	9752104				
	0	Area 3	ND		Area 3	9756893				
STD A	1.40	Area 1	790895	791286	Area 1	9804023	9807280	0.0807	1.3	92.86
		Area 2	792581		Area 2	9810861				
		Area 3	790383		Area 3	9806956				
STD B	8.00	Area 1	2983789	2975634	Area 1	9609456	9608206	0.3097	8.01	100.13
		Area 2	2946532		Area 2	9612581				
		Area 3	2996581		Area 3	9602581				
STD C	16.00	Area 1	5664447	5662504	Area 1	9951818	9948555	0.5692	15.61	97.56
		Area 2	5633204		Area 2	9930567				
		Area 3	5689861		Area 3	9963281				
STD D	28.00	Area 1	9800540	9813654	Area 1	9876462	9866173	0.9947	28.07	100.25
		Area 2	9829568		Area 2	9839561				
		Area 3	9810854		Area 3	9882495				
STD E	40.00	Area 1	13707046	13706216	Area 1	9513119	9510714	1.4411	41.15	102.88
		Area 2	13716598		Area 2	9516596				
		Area 3	13695004		Area 3	9502428				
STD F	56.00	Area 1	18334157	18329215	Area 1	9550847	9532841	1.9227	55.25	98.66
		Area 2	18296532		Area 2	9546981				
		Area 3	18356957		Area 3	9500695				

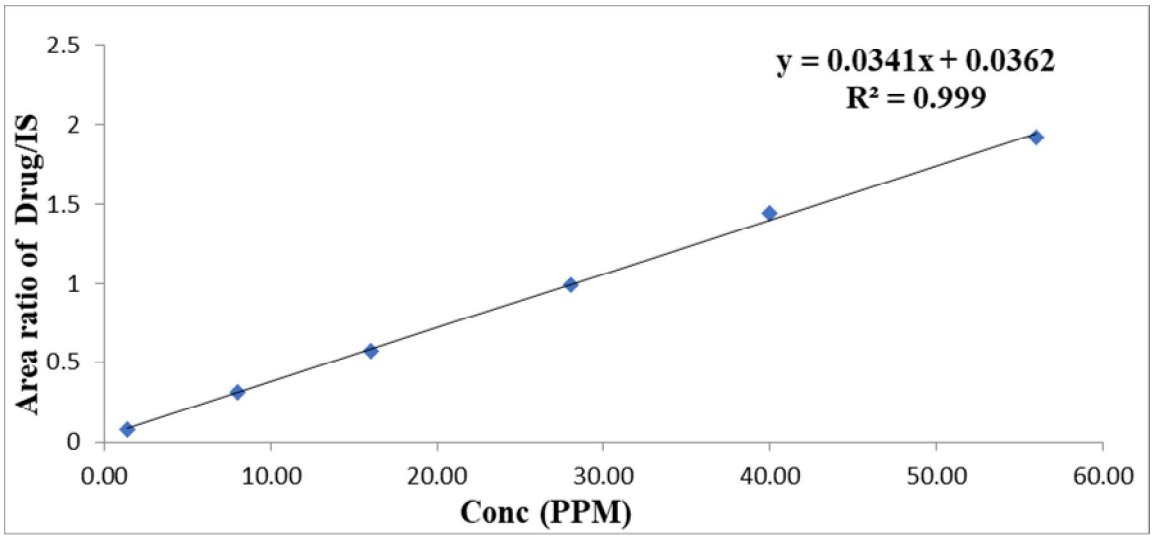

Figure 10: Calibration curve for the linearity of Ibrutinib.

Precision:

The RSD% figures for both precisions were less than 2.0%. The precision of the proposed procedures is shown by the precision research results. The findings of the method precision and system precision studies are shown in Table 2.

Table 2: Precision research results

LEVEL	QC	Recovered concentration	Average Recovered concentration (µg/mL)	% CV
LLOQ	LLOQ 1	1.39	**1.42**	**3.43**
	LLOQ 2	1.43		
	LLOQ 3	1.42		
	LLOQ 4	1.5		
	LLOQ 5	1.36		
	LLOQ 6	1.39		
LQC	LQC 1	4.49	**4.30**	**4.51**
	LQC 2	4.22		
	LQC 3	4.32		
	LQC 4	4.42		
	LQC 5	4.39		
	LQC 6	3.95		
MQC	MQC 1	27.98	**28.27**	**1.92**
	MQC 2	28.26		
	MQC 3	28.62		
	MQC 4	28.26		
	MQC 5	27.45		
	MQC 6	29.04		
HQC	HQC 1	47.59	**45.01**	**3.95**
	HQC 2	42.76		
	HQC 3	44.98		
	HQC 4	44.13		
	HQC 5	44.05		
	HQC 6	46.55		

Accuracy:
The sample solution's mean recovery percentages were 101.07%, 102.04%, 100.96%, and 100.02% for the standard concentration of 1.40, 4.20, 28 and 45 respectively. This implies that ibrutinib in pharmaceutical formulations can be quantified using the techniques mentioned. Accuracy data is shown in Table 3.

Table 3: Accuracy results

SET	QC	Actual conc of QC	Area of Ibrutinib	Area of IS	Area Ratio of Ibrutinib to IS	Recovered conc. (µg/mL)	% Accuracy
SET 1	LLOQ 1	1.40	801469	9652301	0.083	1.39	99.29
	LQC 1	4.20	1876193	9925362	0.189	4.49	106.90
	MQC 1	28.00	9706451	9786294	0.9918	27.98	99.93
	HQC 1	45.00	15673294	9432906	1.6616	47.59	105.76
SET 2	LLOQ 2	1.40	806719	9563027	0.0844	1.43	102.14
	LQC 2	4.20	1776813	9879637	0.1798	4.22	100.48
	MQC 2	28.00	9863410	9852491	1.0011	28.26	100.93
	HQC 2	45.00	14930688	9976539	1.4966	42.76	95.02
SET 3	LLOQ 3	1.40	826749	9832627	0.0841	1.42	101.43
	LQC 3	4.20	1793526	9796539	0.1831	4.32	102.86
	MQC 3	28.00	9805671	9674837	1.0135	28.62	102.21
	HQC 3	45.00	15234260	9686514	1.5727	44.98	99.96
SET 4	LLOQ 4	1.40	862419	9936451	0.0868	1.50	107.14
	LQC 4	4.20	1806751	9675843	0.1867	4.42	105.24
	MQC 4	28.00	9879039	9865381	1.0014	28.26	100.93
	HQC 4	45.00	14685329	9512834	1.5437	44.13	98.07
SET 5	LLOQ 5	1.40	812715	9896748	0.0821	1.36	97.14
	LQC 5	4.20	1793531	9656839	0.1857	4.39	104.52
	MQC 5	28.00	9673008	9935296	0.9736	27.45	98.04
	HQC 5	45.00	15032634	9756294	1.5408	44.05	97.89
SET 6	LLOQ 6	1.40	813859	9786038	0.0832	1.39	99.29
	LQC 6	4.20	1703558	9986426	0.1706	3.95	94.05
	MQC 6	28.00	9913775	9643629	1.028	29.04	103.71
	HQC 6	45.00	16023526	9853627	1.6262	46.55	103.44

Detection and quantitation limit:
When injected 50 ppm of Ibrutinib on HPLC we got about 105 heights by injecting 20µL injection volume.
For accurate and precise quantification, we need the lowest concentration (LLOQ) having at least 3 height so that it can be quantified with accuracy and precision. From 50 PPM we got 105 height so that for 3 height we need to inject 1.4 PPM (1.4 ppm considered as LLOQ) As per above observation 1.4 PPM can be considered as LLOQ. 1.4 ppm is considered as LLOQ. As per EMEA guidelines LLOQ should be NMT 5 times of Cmax. When we consider 1.4 PPM as 5% in that case 100 % is the 28 ppm and ULOQ is Twice of Cmax i.e 56 PPM. So concentration range will be from 1.4 PPM to 56 PPM where 28 PPM will be considered as Cmax. Results were shown in table no 4 as follows.

Table 4: Summary of LLOQ of Ibrutinib and Celecoxib

Plasma Lot	Area of Ibrutinib	Area of IS
LOT 1	819506	9864529
LOT 2	832549	9642608
LOT 3	804251	9946327
LOT 4	798034	9423778
LOT 5	819759	9467957
LOT 6	817637	9836421
Haemolyzed	825791	9736527
Lipemic	830689	9646237

Recovery:
To guarantee accuracy and precision in quantification, the recovery of Ibrutinib using the HPLC technique is evaluated. A validated reverse-phase HPLC method is used, usually with a C18 column that has an

optimized mobile phase composition, like buffer (e.g., formic acid or phosphate buffer) and acetonitrile. Ibrutinib is spiked into a matrix at defined quantities, and then it is extracted and analyzed at a particular wavelength (e.g., 260–280 nm). In order to ensure compliance with ICH requirements, the percentage recovery is computed by comparing the observed concentrations to the spiking levels (usually 98–102% for pharmaceutical formulations). The recovery for Ibrutinib and Celecoxib was found to be 94.02 and 82.76 respectively as shown in table 5 as well as 6.

Table 5: Recovery of Ibrutinib

QC LEVEL	Sample no.	Rec vial Ibrutinib Area	Extracted QC Ibrutinib Area	% Recovery	Mean Recovery	% CV
LQC	LQC 1	1957770	1876193	93.09	**94.02**	**0.99**
	LQC 2	1906521	1776813			
	LQC 3	1986329	1793526			
	Mean	**1950207**	**1815511**			
MQC	MQC 1	10123864	9706451	94.03		
	MQC 2	10653298	9863410			
	MQC 3	10465006	9805671			
	Mean	**10414056**	**9791844**			
HQC	HQC 1	15995171	15673294	94.95		
	HQC 2	16034832	14930688			
	HQC 3	16246830	15234260			
	Mean	**16092278**	**15279414**			

Table 6: Recovery of Internal standard Celecoxib

QC Level	Sample no.	Rec vial IS Area	Extracted QC IS Area	% Recovery	Mean Recovery	% CV
LQC	LQC 1	11844883	9925362	83.07	**82.76**	**0.58**
	LQC 2	11726340	9879637			
	LQC 3	12063054	9796539			
	Mean	**11878092**	**9867179**			
MQC	MQC 1	12021589	9786294	82.21		
	MQC 2	11963507	9852491			
	MQC 3	11673524	9674837			
	Mean	**11886207**	**9771207**			
HQC	HQC 1	11535204	9432906	83.00		
	HQC 2	11683529	9976539			
	HQC 3	11836527	9686514			
	Mean	**11685087**	**9698653**			

Stability:

Stability studies were performed to assess the integrity of the analyte under various conditions, ensuring reliability and accuracy of the bioanalytical method in real-world applications. Three freeze-thaw cycles were conducted by room temperature. The analyte retained 100.20 % of its initial concentration, confirming its stability under freeze-thaw conditions. Bench-top stability at 25°C was assessed for 6 hours, with the analyte showing no significant degradation (101.83 % deviation from nominal value). Stability in the autosampler at 15°C was determined for 24 hours. The mean concentration remained within 100.92 % of the initial value, indicating stability under these conditions. The stability of processed quality control (QC) samples was evaluated by keeping them at room temperature (25°C) for 6 hours. The mean percentage deviation of the analyte concentration from the nominal value was found to be within 100.54 %, which is within the acceptable limit of ±15%. This indicates that the processed samples remain stable under bench-top conditions for up to 6 hours. Stock solution stability was assessed by storing the analyte and internal standard stock solutions at 2–8°C for 24 hours. The percentage difference in analyte concentration between fresh and stored stock solutions was found to be 2.26 %, which is within the acceptance criteria of ±5%. This confirms that the stock solutions are stable for at least 24 hours under the specified conditions. The stability studies demonstrated that Ibrutinib remains stable under all tested conditions, with deviations within the acceptance criteria of ±15%. These findings confirm the robustness of the bioanalytical method for accurate quantification in biological samples. The stability results data shown in table no. 7,8,9,10 and 11.

Table 7: Bench top Stability

Level	QC	Ibrutinib Area	IS Area	Area ratio of analyte to IS	Recovered conc. (µg/mL)	Accuracy	Mean Accuracy	% CV	Overall Accuracy	Overall % CV
LQC	LQC 1	1876193	9925362	0.1890	4.49	106.9	103.41	3.14	101.83	4.26
	LQC 2	1776813	9879637	0.1798	4.22	100.48				
	LQC 3	1793526	9796539	0.1831	4.32	102.86				
HQC	HQC 1	15673294	9432906	1.6616	47.59	105.76	100.25	5.36		
	HQC 2	14930688	9976539	1.4966	42.76	95.02				
	HQC 3	15234260	9686514	1.5727	44.98	99.96				

Table 8: Freeze thaw Stability

Level	QC	Ibrutinib Area	IS Area	Area ratio of analyte to IS	Recovered conc. (µg/mL)	Accuracy	Mean Accuracy	% CV	Overall Accuracy	Overall % CV
LQC	LQC 1	1642531	9753216	0.1684	3.89	92.62	96.83	6.29	100.20	5.33
	LQC 2	1683627	9860429	0.1707	3.95	94.05				
	LQC 3	1763004	9553027	0.1845	4.36	103.81				
HQC	HQC 1	16023493	9856307	1.6257	46.53	103.4	103.58	0.18		
	HQC 2	15763128	9662457	1.6314	46.70	103.78				
	HQC 3	15895635	9763529	1.6281	46.60	103.56				

Table 9: Autosampler Stability

Level	QC	Ibrutinib Area	IS Area	Area ratio of analyte to IS	Recovered conc. (µg/mL)	Accuracy	Mean Accuracy	% CV	Overall Accuracy	Overall % CV
LQC	LQC 1	1793268	9656239	0.1857	4.39	104.52	104.13	0.48	100.92	3.90
	LQC 2	1823631	9841329	0.1853	4.38	104.29				
	LQC 3	1801436	9786531	0.1841	4.35	103.57				
HQC	HQC 1	15023689	9956718	1.5089	43.12	95.82	97.72	2.85		
	HQC 2	14353608	9453694	1.5183	43.39	96.42				
	HQC 3	15642671	9853967	1.5874	45.41	100.91				

Table 10: Stability of processed samples at R.T

Level	QC	Ibrutinib Area	IS Area	Area ratio of analyte to IS	Recovered conc. (µg/mL)	Accuracy	Mean Accuracy	% CV	Overall Accuracy	Overall % CV
LQC	LQC 1	1806751	9675843	0.1867	4.42	105.24	101.27	6.18	100.54	4.48
	LQC 2	1793531	9656839	0.1857	4.39	104.52				
	LQC 3	1703558	9986426	0.1706	3.95	94.05				
HQC	HQC 1	14685329	9512834	1.5437	44.13	98.07	99.80	3.16		
	HQC 2	15032634	9756294	1.5408	44.05	97.89				
	HQC 3	16023526	9853627	1.6262	46.55	103.44				

Table 11: Stock solution stability

Level	QC	Ibrutinib Area	IS Area	Area ratio of analyte to IS	Recovered conc. (µg/mL)	Accuracy	Mean Accuracy	% CV	Overall Accuracy	Overall % CV
LQC	LQC 1	1753074	9860238	0.1778	4.16	99.05	97.30	2.90	97.51	2.26
	LQC 2	1632684	9571452	0.1706	3.95	94.05				
	LQC 3	1713421	9653112	0.1775	4.15	98.81				
HQC	HQC 1	15353206	9798631	1.5669	44.81	99.58	97.72	2.06		
	HQC 2	14353084	9534527	1.5054	43.01	95.58				
	HQC 3	14986453	9716532	1.5424	44.10	98.00				

DISCUSSION

Ibrutinib and Celecoxib were simultaneously quantified in human plasma using an HPLC approach that was developed and optimized for excellent sensitivity, selectivity, and reproducibility. In order to guarantee effective separation and precise quantification of both chemicals, the method development process entailed the careful selection of chromatographic conditions, including the composition of the mobile phase, stationary phase, flow rate, and detection wavelength.

Achieving sufficient separation required careful consideration of the stationary phase selection. Because of its excellent retention properties for lipophilic substances like celecoxib and ibrutinib, a reversed-phase C18 column was used. A combination of acetonitrile and aqueous buffer was used to optimize the composition of the mobile phase in order to improve resolution and peak symmetry while preserving a manageable run time. Isocratic elution offered adequate separation with little baseline noise, therefore gradient elution was investigated but determined to be superfluous. A UV detector set at an ideal wavelength was used for detection in order to guarantee that both chemicals showed enough absorption. The limit of detection (LOD) and limit of quantification (LOQ), which were well within the permissible range for bioanalytical applications, were used to assess the method's sensitivity. Excellent correlation coefficients ($R2 > 0.99$) were shown by the calibration curve's linearity for both medications, suggesting a reliable analytical technique. For plasma sample preparation, protein precipitation was used to guarantee efficient extraction with little matrix interference. Acetonitrile showed the best recovery and the least amount of co-elution with endogenous plasma components among the solvents studied. Ibrutinib and Celecoxib extraction recovery rates were found to be reliable and consistent over a range of concentrations, demonstrating the effectiveness of the sample preparation technique.

Regulatory criteria for the validation of bioanalytical methods were followed in the validation process. Studies on precision and accuracy showed that intra- and inter-day variations were within allowable bounds. Both medications' stability in plasma under various handling and storage circumstances was evaluated as well, and the results showed no discernible deterioration over time. Furthermore, matrix effects were assessed to verify that plasma components did not impede the detection of the analyte, guaranteeing dependability in practical bioanalysis.

CONCLUSION

Ibrutinib and Celecoxib can be quantified in human plasma using the established HPLC method, which is easy to use, dependable, and appropriate for bioanalytical applications. It offers a reliable method for clinical research involving these two drugs, pharmacokinetic studies, and therapeutic medication monitoring. Future studies involving Ibrutinib and Celecoxib in plasma samples will benefit greatly from the method's sensitivity, accuracy, and reproducibility.

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