DEVELOPMENT AND VALIDATION OF A SENSITIVE SPECTROFLUORIMETRIC METHOD FOR THE DETERMINATION OF IBRUTINIB**

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A reproducible, sensitive, and cost-effective spectrofluorimetric method has been developed for quantification of the drug ibrutinib in bulk and in its oral capsule formulation. The method was validated in accordance with the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use guidelines with respect to linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. The method was found to be extremely sensitive, and excellent linearity was noted within the concentration range 0.1–7.5 μg/mL with a correlation coefficient (r^2) of 0.9961. The LOD and LOQ values for the proposed method were found to be 0.224 and 0.680 μg/mL, respectively. Excellent recoveries of the drug were observed from the marketed capsule formulation by the proposed method (97.59, 1.42% relative standard deviation).

Keywords: *ibrutinib, spectrofluorimetry, validation, analysis.*

РАЗРАБОТКА И ВАЛИДАЦИЯ ЧУВСТВИТЕЛЬНОГО СПЕКТРОФЛУОРИМЕТРИЧЕСКОГО МЕТОДА ОПРЕДЕЛЕНИЯ ИБРУТИНИБА

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Разработан воспроизводимый, чувствительный и экономичный спектрофлуориметрический метод для количественного определения лекарственного средства ибрутиниба в нерасфасованной и пероральной капсульной формах. Проверка метода в соответствии с руководящими принципами ICH показала его высокую чувствительность, отличную линейность в диапазоне концентраций 0.1–7.5 мкг/мл с коэффициентом корреляции r^2 = 0.9961, LOD и LOQ 0.224 и 0.680 мкг/мл. Извлечение лекарственного средства из капсульной композиции по предлагаемому способу 97.59, 1.42 % RSD.

Ключевые слова: *ибрутиниб, спектрофлуориметрия, валидация, анализ.*

Introduction. Ibrutinib (Ibrunat* by Natco Pharma), 1-[(3R)-3-[4-amino-3-(4-phenoxyphenyl)-1H-pyrazolo[3,4-d]pyrimidin-1-yl]piperidin-1-yl]prop-2-en-1-one (CAS No. 936563-96-1) is a novel small molecule covalent inhibitor of the enzyme Bruton's Tyrosine Kinase (BTK) [1, 2], approved by the FDA for the treatment of mantle cell lymphoma and chronic lymphocytic leukemia. An extensive literature survey related to the analytical method development for ibrutinib retrieved several reports on chromatographic methods (high-performance liquid chromatography [HPLC], ultrahigh-performance liquid chromatography [UHPLC], liquid chromatography–mass spectrometry [LC-MS]/MS) for the analysis of pure ibrutinib, in combination with other drugs or in the presence of various impurities or its degradation products. Several methods have focused on the determination of the drug in biological matrices. These include simultaneous determination of

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lenalidomide, ibrutinib, and its active metabolite PCI45227 in rat plasma by LC-MS/MS [3], bio-analytical assay for ibrutinib and its dihydrodiol metabolite in human and mouse plasma [4], a UHPLC-tandem mass spectrometry procedure for the quantification of ibrutinib and its active metabolite PCI-45227 in cerebrospinal fluid [5], bioanalysis of ibrutinib and its dihydrodiol and glutathione cycle metabolites by LC-MS/MS [6], and simultaneous quantitative determination of anlotinib, ceritinib, and ibrutinib in rat plasma [7]. The determination of plasma ibrutinib concentrations has been reported using the HPLC [8, 9] and LC-MS/MS [10, 11] techniques. Studies related to the estimation of ibrutinib in bulk or in drug products, in the presence of its process-related impurities [12] and degradation products [13–15], have also been reported as stability-indicating HPLC methods. Vajjha et al. [13] have identified two novel degradation products from this drug. Another novel stability-indicating method of separation and identification of genotoxic impurities using RP-HPLC/PDA and QDa mass detectors has been recently reported [16]. Some reverse-phase liquid chromatographic methods have been recently reported for the determination of ibrutinib in bulk and in its pharmaceutical dosage form [17–21]. The literature review did not retrieve any reports on the spectrofluorimetric method of determination for ibrutinib. Hence, the present study was envisaged to develop and validate a simple, sensitive spectrofluorimetric method for the quantification of ibrutinib in bulk and in its marketed capsule formulation (Ibrunat* by Natco Pharma). The developed method was validated as per various parameters outlined in the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline Q2(R1) [22].

Experiments. Ibrutinib (Batch number – IWA17000) was kindly gifted by Cipla Pharma Ltd. (Mumbai, India). All chemicals and materials were of analytical grade and were purchased from Merck India Pvt. Ltd., Mumbai. All solutions were freshly prepared using triple distilled water obtained from a Milli-Q plus purification system Millipore (Bradford, USA). Ibrunat® capsules (140 mg of ibrutinib per capsule; Natco Pharma, Hyderabad, India) were purchased from the market.

All the glassware including a volumetric flask, a pipette, a measuring cylinder, beakers, and test tubes was of Class A grade and were purchased from Borosil (Mumbai, India). Absorption and emission spectra were recorded using a Hitachi (Tokyo, Japan) spectrofluorometer F2500 with a scanning speed of 300 nm/min, 10-mm matched quartz cells, and a resolution of 2.5 nm. A melting point apparatus (model T0603160; EIE Instruments Pvt. Ltd., Ahmedabad, India) was used for the determination of the melting point of ibrutinib. A digital pH meter (model GC7252101B; Eutech Instruments, Paisley, UK) was used to adjust the pH of the buffer solution.

A standard stock solution of ibrutinib (1000 μg/mL) was prepared daily by dissolving 10.0 mg of ibrutinib in 10 mL of the solvent (methanol). This was diluted 1 in 10 to obtain a stock solution (100 μg/mL). The working standard solutions ranging from 0.1 to 10.0 μg/mL of ibrutinib were prepared by serial dilutions of the working standard solution with methanol, and the test tubes were kept stoppered to avoid evaporation of the solvent.

Preparation of calibration curves for ibrutinib. The excitation and emission spectra for the working standard solutions of ibrutinib ranging from 0.1 to 10.0 μg/mL were recorded over the wavelength ranges 200–400 and 400–800 nm, respectively. The maximum absorbance of the drug (λ_{max}) was observed at 289 nm, which was selected as the excitation wavelength, whereas 397 nm was selected as the emission wavelength for the measurement of the fluorescence intensity. The optimized method was validated with respect to various parameters outlined in the ICH guideline Q2(R1).

Recovery studies with the marketed formulation of ibrutinib. A powder weight equivalent to 50 mg of ibrutinib (Inbrunat® capsules; label amount 140 mg ibrutinib per capsule; Natco Pharma, Hyderabad, India) was sonicated with methanol to prepare 100 mL of the stock solution (500 μg/mL). The solution was diluted (1 in 10) to prepare the working standard solution (50 μg/mL). This solution was diluted (1 in 20) and analyzed for the drug content in triplicate, by the developed method.

Results and discussion. We have tried to develop and validate a sensitive spectrofluorimetric method for the analysis of ibrutinib in bulk in its capsule formulation. Ibrutinib contains a pyrazolo[3,4-d]pyrimidine moiety linked to a diphenyl ether moiety at 3-position (Fig. 1), having a conjugated system of double bonds placed in a planar stereochemical orientation, which should impart fluorochrome properties to the molecule. It is a practically insoluble drug with a pK_a value of 3.74 (aqueous solution using methanol as a co-solvent) [23]. The drug is soluble in solvents such as methanol, ethanol, DMSO, and DMF. It is sparingly soluble in aqueous buffers and may be adequately solubilized with the aid of organic solvents such as methanol, ethanol, or DMSO [24]. A systematic analysis of the fluorescence characteristics of the drug revealed that ibrutinib possesses good native fluorescence in methanol, ethanol, and acidic media. In preliminary studies, drug

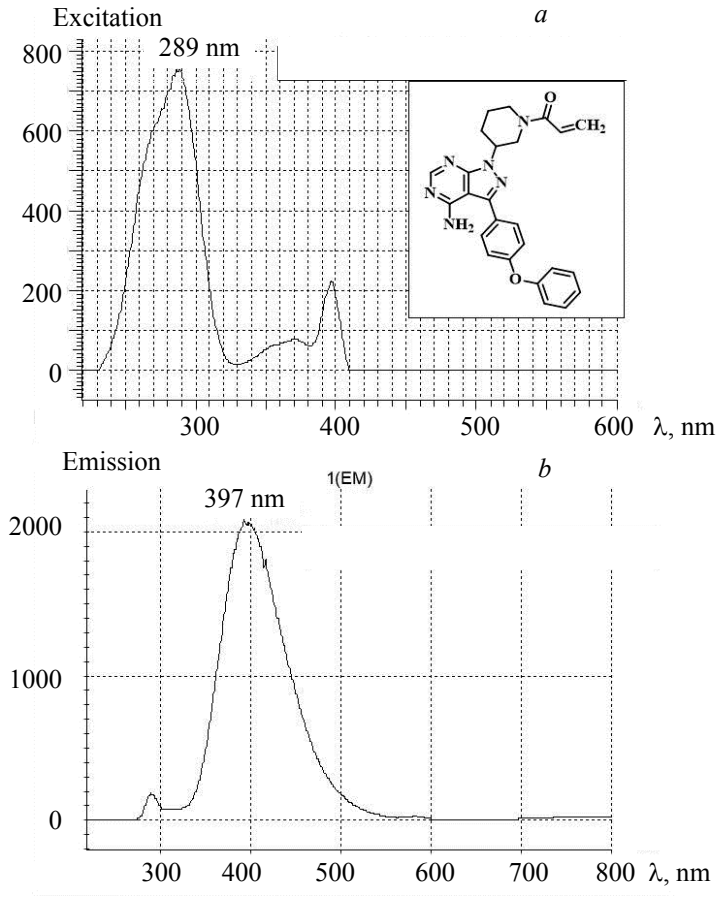

Fig. 1. Excitation (a) and emission (b) scans of ibrutinib in methanol.

TABLE 1. Validation Parameters for the Proposed Method

Accuracy	Conc. of drug taken, μg/mL	Conc. of std. Added, μg/mL	Spiked conc., μg/mL	Calculated concentration (μg/mL)±SD, %RSD	%Recovery
	5.0	10.0	2.5	2.55±0.025; 0.98	102.03
	5.0	15.0	5.0	5.23±0.011; 0.21	104.68
	5.0	25.0	10.0	10.63±0.23; 0.21	106.32
Precision	Conc. taken, μg/mL	Calculated concentration (μg/ml) ± SD, %RSD			
		Intra-day (n = 6)		Inter-day (n = 3)	
	1.5	1.50±0.021; 1.4		1.20±0.013; 1.08	
	4.0	4.04±0.038; 0.95		4.03±0.0412; 1.02	
	7.5	7.55±0.09; 1.19		7.55±0.034; 0.456	
Linearity	Range, μg/mL	Slope	Intercept	Coefficient of correlation r^2	
	0.1–7.5	68.58	26.29	0.993	
LOD, μg/mL	0.224				
LOQ, μg/mL	0.680				

solutions were prepared in various acidic buffers (with the aid of methanol), and the corresponding fluorescence intensities were measured. The poor solubility of the drug made extensive exploration of its fluorescence characteristics in other buffer types, such as phosphate buffers, nonfeasible. The fluorescent intensities for the drug solution (5 μg/mL) in an ammonium acetate buffer (pH 3.8) and an ammonium formate buffer (pH 2.8–4.8) were found to be low (ranging from 50 to 60 FU). Further, fluorometric testing was done in various solvents such as methanol, ethanol, and acetonitrile. Considering the UV absorption and solubility characteristics of the drug/solvent and the respective fluorescence intensities in various solvents and buffers,

methanol was selected as an appropriate solvent that returned good fluorescence intensities at the selected low drug concentrations. The fluorescence intensity of the drug solutions/stress degraded solutions was determined taking 289 and 397 nm as the excitation and emission wavelengths respectively against the reagent blank. Figure 1 shows the excitation and emission spectra of ibrutinib in methanol. The developed method was validated with respect to linearity, range, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ), and robustness. The various validation parameters are summarized in Table 1.

A strictly linear relation was observed between the fluorescence intensity and the concentration of ibrutinib within the concentration range 0.1–7.5 μg/mL. Fluorescence intensities in the linearity studies for the proposed method are shown in Table 2. The calibration curve can be described by the equation $y = 68.58x + 26.29$ ($n = 3$, $r^2 = 0.993$) (Fig. 2).

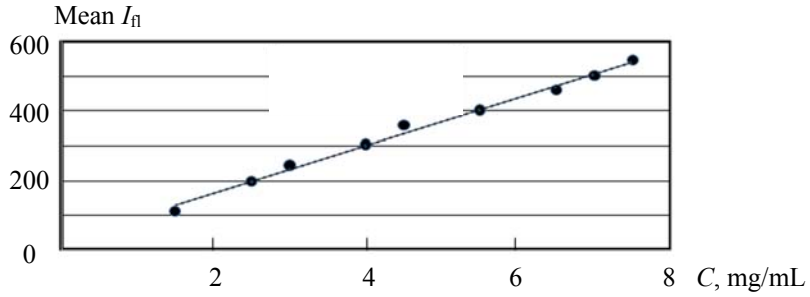

Fig. 2. Calibration plot for ibrutinib with the proposed spectrofluorimetric method.

TABLE 2. Fluorescence Intensities in Linearity Studies for the Proposed Method

Drug conc., μg/mL	Set I	Set II	Set III
1.5	112.2	112.1	112.2
2.5	197.4	197.2	197.8
3.0	243.7	243.7	243.2
4.0	308.9	289.9	308.9
4.5	357.7	357.2	357.2
5.5	409.6	383.6	408.9
6.5	471.8	471.8	431.8
7.0	500.2	501.2	500.0
7.5	544.2	544.6	544.7
Slope	69.31	69.18	67.25
Intercept	26.06	21.74	31.9
r^2	0.994	0.991	0.984

The accuracy of the proposed method was assessed by preparing different concentrations of the drug for analysis, from independent stock solutions. Further assessment of the accuracy of the developed method was done by spiking the excess drug (50, 100, and 150%) to pre-analyzed drug solution samples (5.0 μg/mL). Accuracy was determined as the mean % recovery of the spiked drug concentration (Table 3).

Intra-day precision was investigated by analyzing varying concentrations of ibrutinib in six independent replicates on the same day. Inter-day precision was ascertained from similar determinations carried out on three consecutive days (Table 4). The method was found to be sufficiently precise with %RSD for the intra-day and inter-day precision not exceeding 1.30 and 1.80%, respectively. The calculated intra-day and inter-day precision of the proposed method of analysis are given in Table 1, and no significant variation in the calculated drug concentration was observed on any day. This showed that the method was sufficiently precise for determining the drug concentrations.

The LOD and LOQ were calculated using the formulae ($3.3\sigma/s$) and ($10\sigma/s$) respectively, where σ is the standard deviation of the response (calculated from the standard deviation of the intercept) and s is the slope of the calibration curve. The slopes and intercepts of calibration plots for three sets of fluorescence intensities taken from the linearity studies (Table 5) were taken for the calculation of LOD and LOQ values. The LOD and LOQ were found to be 0.224 and 0.680 μg/mL, respectively. Further, solutions of the drug having

concentrations corresponding to the LOD and LOQ values were prepared and analyzed six times (n = 6). The %RSD for the results corresponding to both the LOD and LOQ were found to be less than 2.8%.

TABLE 3. Accuracy Data for the Proposed Method

Spiked drug conc., μg/mL [a]	Fluorescence intensity [b]	Spiked concentration (μg/mL)±SD, %RSD [c]	
		Calculated [d]	% Recovery
2.5	201.23	2.55±0.025; 0.98	102.03
5.0	385.26	5.23±0.011; 0.21	104.68
10.0	755.46	10.63±0.23; 0.21	106.32

[a] Drug solution (5.0 μg/mL) mixed with equal vol. of std. drug solutions with conc. 10.0, 15.0, and 25.0 μg/mL.
[b] Calculated as fluorescence intensity (spiked solution) − fluorescence intensity (original drug solution)
[c] Calculated as 100×SD/mean.
[d] Calculated as a mean of three measurements (n = 3).

TABLE 4. Fluorescence Intensities in the Intra-day and Inter-day Precision Studies with Pure Drug Ibrutinib

Conc., μg/mL	*Intra-day*					
	Set 1	Set 2	Set 3	Set 4	Set 5	Set 6
1.5	112.2	112.1	122.2	112.4	112.6	112.2
4.0	308.9	289.9	308.9	301.1	301.1	301.1
7.5	544.2	544.6	544.7	544.1	544.2	544.7

	Inter-day								
	I			II			III		
	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
1.5	112.2	112.1	112.2	112.3	112.2	112.2	115.6	115.2	112.3
4.0	308.9	289.9	308.9	308.2	299.2	289.1	307.1	307.1	307.2
7.5	544.2	544.6	544.7	544.4	544.2	543.2	544.1	544.0	543.5

TABLE 5. Data for Calculation of LOD and LOQ

Set	Set I	Set II	Set III	Mean	Std. dev.
Slope	69.31	69.18	67.25	68.79 [a]	0.5827
Intercept	26.06	21.74	31.09	26.29	4.6794 [b]

[a] Mean slope of the calibration plot.
[b] Standard deviation of the response.

The method was found to be robust as no significant changes in the fluorescence intensity were observed after carrying out deliberate changes in the method variables including the excitation wavelength, emission wavelength, and analyst performing the study. The %RSD in all cases was found to be less than 1.70%. The results from the robustness studies are shown in Table 6.

TABLE 6. Robustness of the Proposed Method

Parameter	Change	Fluorescence intensity			Mean	SD	%RSD
Optimized conditions	NA	124.8	125.9	126.9	125.86	1.05	0.83
Excitation λ_{max} (291 nm)	295	110.9	112.6	109.9	111.13	1.36	1.22
Emission λ_{max} (359 nm)	365	115.8	116.8	114.9	115.83	0.95	0.82
Analyst I	Analyst II	123.9	124.6	126.4	124.96	1.28	1.03

The solutions were found to be stable for up to 8 h with %RSD values lower than 2.0%.

The data from the recovery studies of the drug in the marketed capsule formulation of ibrutinib (Inbrunat® capsules; label amount: 140 mg of ibrutinib per capsule; Natco Pharma, Hyderabad, India) are

shown in Table 7. The marketed formulation showed recoveries ranging from 96.14 to 98.92% with low RSD% values (1.43%).

TABLE 7. Recovery Studies with the Drug Formulation

Label claim, mg	Recovery, mg	%Recovery
140	138.5	98.92
140	136.8	97.71
140	134.6	96.14
Mean	136.63	97.59
SD	1.96	1.39
%RSD	1.43	1.42

Conclusions. A rapid and reliable spectrofluorimetric method has been proposed for the determination of ibrutinib in bulk as well as in its marketed capsule formulation. The method was validated for various parameters including sensitivity, reproducibility, precision, accuracy, robustness, and solution stability for ≥8 h. Good recoveries of the drug from bulk samples and drug formulation suggest that the method is suitable for routine drug analysis without interference from the formulation excipients.

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