RESEARCH ARTICLE

Quantification of Bictegravir and its impurities: A novel RP-UPLC Approach for Development and Validation

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ABSTRACT

A new ultra-pressure liquid chromatography (UPLC) method was developed for accurately and reliably quantifying impurities in Bictegravir. The method utilized Waters Acquity HSSC18 column (50mm x 2.1mm, 1.8 µ) at room temperature. The mobile phase consists of Acetonitrile and Buffer (0.1 percent formic acid) in 80:20 and pumped at a flow rate of 0.2 mL/min. This optimized method achieves exceptional separation of Bictegravir and its impurities with excellent resolution at 281 nm. The method's accuracy is demonstrated by a % recovery range for Bictegravir and its impurities between 99.1% and 100.3%. The correlation coefficient (R^2) for BIC and its impurities is consistently above 0.999, indicating high linearity. The method's robustness was confirmed by its stability against variations in flow rate and mobile phase ratio. Additionally, forced degradation studies confirmed the stability of Bictegravir, highlighting the method's reliability. Overall, developed UPLC method is precise, accurate, robust, and linear, making it suitable for routine analysis of BIC-related substances in quality control laboratories at manufacturing sites during commercial production.

Keywords: Bictegravir, Impurity, HSS C18 Column, Stability Indicating, Resolution.

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INTRODUCTION

Analytical methods for impurity profiling are critical to ensuring pharmaceutical compounds' safety, efficacy, and quality.[1] These approaches, which are often developed using advanced chromatographic techniques like ultra-pressure liquid chromatography (UPLC) and high-performance liquid chromatography (HPLC), were intended to identify and quantify impurities in a medicinal molecvule precisely.[1-3] These procedures must meet major regulatory requirements, such as those specified by the International Council for Harmonisation (ICH Q3A and 3B), with high specificity, sensitivity, precision, accuracy, and robustness. The goal is to ensure that impurities are regularly and reliably detected at trace levels, ensuring the quality and safety of pharmaceuticals.[2-4] Bictegravir (BIC) is a second-generation integrase strand transfer inhibitor (INSTI) that is licensed to treat HIV when combined with emtricitabine and tenofovir alafenamiden.5 This powerful antiviral medication acts against both wild-type forms of HIV and those that are resistant to first-generation INSTIs.[6,7] BIC has the molecular formula $C_{21}H_{18}F_3N_3O_5$, and its bicyclic core and fluorine atoms improve its pharmacokinetic characteristics. It operates by binding to the active site of the HIV integrase enzyme, preventing the strand transfer step required for viral DNA integration into the host genome.[6-8] This inhibition prevents virus replication, lowering the viral burden in patients.[6] The potent impurities of BIC were shown in Figure 1.

During the literature study, several assay methodologies for assessing BIC in combination with other antiretroviral drugs emerged. Several published methods use Liquid Chromatography and Mass Spectrometry (MS) techniques.[9-18] Few analytical methods (HPLC and LC-MS) are available in the literature for analysis of BIC alone or in combination with other antiviral drugs.[9-18] Among them, one significant method is the detection and quantification of related compounds using HPLC and MS.18 However, a thorough review of the available literature showed a substantial gap: no UPLC method has been developed or validated, especially for the measurement for finding the impurities (IMP) and relative compounds of above-mentioned drug BIC.

Given the lack of a UPLC approach, there is a high need to develop a robust, accurate, and precise UPLC-based assay for measuring impurities in BIC. The above strategy will comply with the International Council for Harmonisation (ICH) principles, guaranteeing that it fulfills stringent accuracy, precision, specificity, and repeatability standards.

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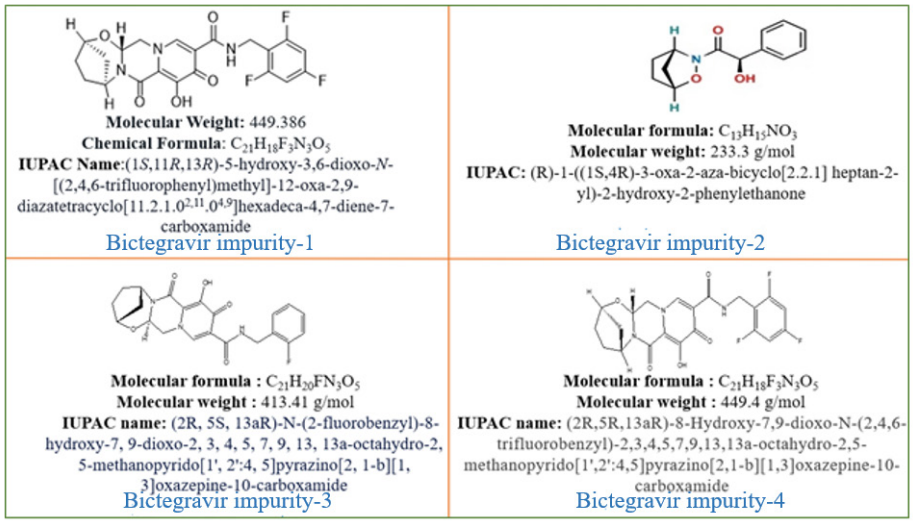

Figure 1: Chemical structures of Bictegravir and its impurities

The development of such a procedure would improve BIC quantitative abilities and serve as a trustworthy tool for general testing in quality control laboratories.

MATERIALS AND METHODS

BIC in its pure form and its impurities were gifted as samples from Glenmark Pharmaceuticals in Mumbai, India. The remaining chemicals and reagents used in the current research work were purchased from Worli, Mumbai, India. The proposed method was executed using an Agilent 1290 Infinity II UPLC instrument coupled with Empower 2.0. A nylon filter of 0.45µ was used to filter the mobile phase and solutions used in the current study.

Method Development:

Trial and error approach was performed using Waters BEH C18 (100 x2.1 mm, 1.7 µ) column and ACN: 0.1 % v/v TFA mobile phase in 70:30; 50:50; 60:40 v/v proportions, respectively, but symmetrical peaks were not achieved. Later trials were done on HSS C18 (50 x 2.1mm, 1.8 µ) using mobile phase of ACN: 0.1 % formic acid in 70:30 and 50:50 proportions respectively, but the peak resolution is low. Ultimately an optimized condition was attained on Waters acuity HSS C18 (50 x 2.1mm, 1.8 µ) column and ACN: 0.1 % v/v formic acid (80:20 v/v).

Chromatographic Conditions:

The current RP-UPLC method was developed by using Agilent 1290 Infinity II UPLC coupled with the PDA detector and auto sampler. Effective separation of BIC and its three related impurities have been achieved by following chromatographic conditions comprising of Acquity HSS C18 (50 x 2.1mm, 1.8 µ) column, mobile phase of ACN: 0.1% formic acid in 80:20 with a flow rate of 0.2mL/min, isocratic elution mode and finding wavelength of 281nm. Room temperature was maintained in the injection port and in the analytical column. Mobile phase was used as diluent. The BIC and its impurities 2, 3, and 4 were eluted at 5.4, 2.0, 2.98, and 4.0 minutes, respectively, with good resolution (Figure 2).

Standard Stock Solution Preparation (5000 µg/mL)

A stock solution of BIC was made by transferring exactly 500 mg to a 100 mL volumetric flask. The BIC was dissolved by adding approximately 7 mL of diluent and sonicating the mixture for around 15 minutes. After dissolving, the solution was diluted using diluent in order to achieve final concentration of 5000 µg/mL of BIC.

Impurity Stock Solution Preparation

Accurately transfer 5mg each of BIC impurities 2, 3, and 4 to a 100 mL volumetric flask. Add 70 mL of diluent and follow sonication for 20 minutes to ensure complete dissolution. Dilute to the mark using diluent to get a final concentration of 50 µg/mL for each impurity.

Preparation of Spiked Solution

In a 50 mL volumetric flask, 5 mL of the BIC stock solution was transferred. Subsequently, 30 mL of diluent was added, followed by 5 mL of the impurity stock solution. The solution was then diluted to 50 mL with the diluent. The resulting solution, containing BIC at a concentration of 50 µg/mL and impurities 2, 3, and 4 each at a concentration of 5µg/mL, was filtered with a 0.22 µm syringe filter to eliminate any particles. It is considered a 100% level solution.

Method Validation

In compliance with the ICHQ2 (R2) guidelines, the developed method has been validated [19].

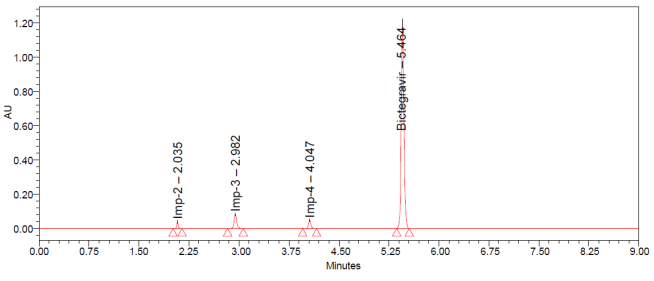

Figure 2: Optimized chromatogram for BIC and its impurities

System Suitability

It was performed by injecting a spiked standard solution containing 500 µg/mL of BIC and 50µg/mL of each stated impurity in 6 subsequent injections. The % RSD of the peak areas and mean of the remaining parameters (Tailing factor, theoretical plates) were computed to confirm the method's suitability.

Accuracy

It was done by measuring the recovery of BCT and its impurities in 50, 100, and 150% levels of spiked solution. The amount recovered, and % RSD of BCT and its impurities were computed from the data obtained.

Precision

The precision technique was calculated by injecting six freshly prepared homogenous spiked sample aliquots containing concentrations of 500 µg/mL of BIC and 50 µg/mL of BIC Imp (2, 3, and 4). The percent RSD was assessed of RT, peak area and relative retention time (RRT). The intermediate precision of the current method was executed in the same fashion for three continuous days (two times per day).

Specificity

It is the ability to measure the response of any intended substances in the presence of other analytes. The specificity of the procedure is done by injecting subsequent injections of blank Bictegravir spiked with impurities solution and Forced degradation solution of BIC standard solution. The interference from the other substances (Degradants and at the RT of BIC and stated impurities was checked

Sensitivity

In analytical procedures, the signal-to-noise ratio (S/N) is critical for defining the limits of detection (LOD) and quantification (LOQ). The LOD requires a S/N ratio of at least 3:1 to ensure that the signal can be distinguished from background noise. The LOQ requires a S/N ratio of at least 10:1, which ensures accurate analyte quantification. These ratios contribute to determining the sensitivity and reliability of the analytical procedure.

Linearity

Six linearity aliquots of BIC (125-750 µg/mL) and six aliquots of Impurity-2, Impurity-3, and Impurity-4 (1.25-7.5µg/mL) were precisely prepared and injected into the analytical devices. These aliquots aimed to test the method›s linearity over a range of concentrations. A calibration curve was produced for every single substance to determine the slope, Y-intercept, and coefficient of determination (R^2).

Robustness

The flow rate (±0.02 mL/min) and organic phase composition (±10% or 8 parts) were deliberately modified from the recommended levels to determine their impact on system suitability characteristics and % RSD. The findings confirmed the method's robustness by examining how modifications affected the system's performance and consistency.

Forced Degradation Studies

The forced degradation investigations assessed BIC stability in different stress environments, including acidic, basic, oxidative, thermal, and photolytic. The findings aided in understanding the degradation pathways and identifying probable degradation products, verifying the method's robustness and stability-indicating capacity to measure BIC and its impurities, and ensuring the pharmaceutical product's quality and efficacy.20 A BIC standard stock solution was prepared by weighing 50 mg of the BIC standard and transferring it to a 10 mL volumetric flask. After adding 7 mL of diluent, the mixture was subjected to sonication for 15 minutes to achieve full dissolution before being diluted to the desired level with diluent. For acid degradation investigations, 1-mL of stock solution was combined with 1-mL of 1N HCl in a 10 mL volumetric flask and left at room temperature for 30 minutes before adding 1-mL of 1N NaOH and diluting to the mark with diluent. For base degradation, 1 mL stock solution solubilized with 1 mL of 1N NaOH and kept aside at room temperature for 30 minutes before adding 1 mL of 1N HCl and diluting to the mark with diluent. For peroxide degradation, 1-mL of the stock solution was combined with 1-mL of 10% H_2O_2, left aside in room temperature for 30 minutes, and then diluted to the mark using diluent. For thermal degradation, 70 mg of BIC standard was heated in a hot air oven at 105°C for six hours. Following the exposure, 50 mg of the thermally exposed standard was transferred to 10 mL volumetric flask and diluted to the mark using diluent. The solution was diluted from 1-10 mL with diluent to reach a concentration of 500 µg/mL. For photolytic degradation, 1mL of the stock solution was shifted to a 10 mL volumetric flask, diluted up to the mark using diluent, and left in UV light for 6 hours.

RESULTS AND DISCUSSION

System Suitability

These parameters were reproducible (Table 1), and the computed % RSD was less than 2.0 for BIC and its impurities.

Bictegravir and its impurities have computed R^2 value of 0.999 over a concentration of 125 - 750µg/mL (Bictegravir) and

Table 1: Bictegravir system suitability results and impurities with optimized conditions

Compound	Retention time (min)*	RRT (min)*	Area count*	No of plates*	Tailing factor*	Resolution*	%RSD*
BIC Imp-2	2.035	0.38	185248	3286	1.01	-	0.8
BIC Imp-3	2.982	0.54	336521	8254	1.08	5.01	0.9
BIC Imp-4	4.047	0.75	216859	4972	1.02	6.35	1.1
BIC	5.464	-	2547184	15896	1.09	10.57	0.8

n-mean of six replicates, *= n=6

S.No	Bictegravir		Impurity-2		Impurity -3		Impurity -4	
	Conc. (µg/mL)	Area count	Conc. (µg/mL)	Area count	Conc. (µg/mL)	Area count	Conc. (µg/mL)	Area count
1	125	6285214	1.25	45857	1.25	86984	1.25	55623
2	250	12985467	2.50	93251	2.50	178458	2.50	105847
3	375	18805694	3.75	139958	3.75	257485	3.75	159854
4	500	25846725	5.00	184758	5.00	335879	5.00	211854
5	625	31845724	6.25	227854	6.25	415623	6.25	267548
6	750	38100521	7.50	277623	7.50	501248	7.50	313256

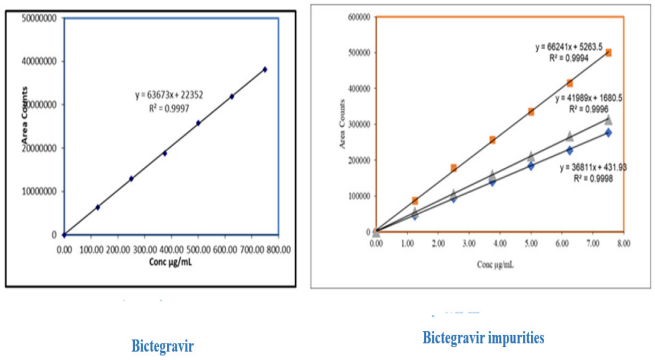

Figure 3: Calibration curves of Bictegravir and its impurities

1.25 -7.5 µg/mL (Impurities) (Figure-3), indicating significant linearity within the range. The Bictegravir mean percentage recovery and its impurities in spiked standard solutions was 100% ± 2, showing method accuracy (Table 2). The % RSD values of peak area responses from six consecutive injections of spiked standard solutions ranged from 0.72 to 1.31 (Table 3), indicating the new method's precision. Bictegravir and it's LOD and LOQ) were significantly less, with good sensitivity (Table 4). Furthermore, purposeful and modest modifications in method parameters had no significant effect on performance, with % RSD values are within ICH acceptable limits (Table 5), demonstrating the procedure's robustness. Interference at RT Bictegravir and its impurities were not observed with the RT of blank, degradants, or placebo, demonstrating the method's specificity (Figure 4).

Forced Degradation

The impurities were clearly separated (Figure 5), and the % degradation obtained (Table 6) was recorded along with the system suitability parameters. The purity angle was less than

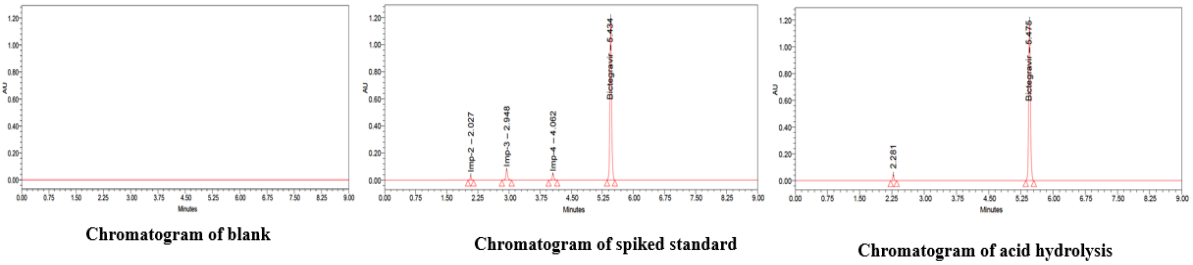

Figure 4: Specificity of optimized method

Table 2: Bictegravir impurities and accuracy results

Level (%)	*Bictegravir*		*Impurity-2*		*Impurity-3*		*Impurity-4*	
	*Amount recovered (µg/mL)**	*Recovery (% w/w), % RSD**	*Amount recovered (µg/mL)**	*Recovery (% w/w), % RSD**	*Amount recovered (µg/mL)**	*Recovery (% w/w), % RSD**	*Amount recovered (µg/mL)**	*Recovery (% w/w), % RSD**
50	24.793	99.2, 0.64	2.496	99.2, 1.45	2.483	100.6,2.8	2.490	100.3,0.83
100	50.100	100.2,0.6	4.973	98.4, 2.30	4.956	97.8, 2.00	4.960	97.5, 0.88
150	74.3	99.1, 0.25	7.400	99.8, 0.56	7.400	99.6, 1.16	7.400	99.5, 0.20

n-Mean of three replicates, *= n=6

Table 3: Intermediate precision and method results

Compound	*RT (min)**	*RRT (min)**	*Peak area ± % RSD**	*Mass recovery (% w/w) **
Method precision				
Bictegravir	5.54	-	25678480 ± 0.54	-
IMP-II	2.08	0.38	185948 ± 1.46	0.72
IMP-III	2.97	0.54	335398 ± 0.68	1.31
IMP-IV	4.08	0.73	214927 ± 0.66	0.84
Intermediate precision				
Bictegravir	5.49	-	25666687 ± 0.82	-
IMP-II	2.07	0.38	185766 ± 0.78	0.72
IMP-III	2.98	0.54	335149 ± 0.86	1.31
IMP-IV	4.09	0.74	214749 ± 1.08	0.84

n-Mean of six replicates, *= n=6.

Table 4: The values of LoD and LoQ

Compund	*LoD (µg/mL)*	*LoQ (µg/mL)*
Bictegravir	0.021	0.502
Impurity-II	0.005	0.01
Impurity-III	0.005	0.01
Impurity-IV	0.005	0.01

Table 5: Robustness results of BIC and its impurities

Compound	*Parameter variation*		*RT (min)**	*Area count, % RSD**	*No. of plates**	*Tailing factor**	*Resolution**
Bictegravir	Flow rate (mL/min.)	0.18	5.707	22372768, 0.62	15960	1.05	8.54
		0.20	5.464	25471846, 0.80	15896	1.09	10.57
		0.22	5.256	27699046, 0.76	15762	1.03	7.53
	Mobile phase (8 parts)	72:28	5.912	20501611, 0.81	16030	1.04	7.33
		80:20	5.464	25471846, 0.80	15896	1.09	10.57
		88:12	5.094	29490464, 0.50	14548	1.06	7.52
Impurity-II	Flow rate (mL/min.)	0.18	2.365	1546697, 1.57	3353	1.07	-
		0.20	2.035	185248, 0.80	3286	1.01	-
		0.22	1.88	207615, 0.60	3135	1.03	-
	Mobile phase (8 parts)	72:28	2.52	138193, 1.25	3435	1.06	-
		80:20	2.035	185248, 0.80	3286	1.01	-
		88:12	1.78	226356, 0.49	3031	1.06	-
Impurity-III	Flow rate (mL/min.)	0.18	3.08	326187 ,0.67	8357	1.05	2.63
		0.20	2.982	336521, 0.90	8254	1.08	5.01
		0.22	2.826	343067, 0.56	8140	1.04	4.65
	Mobile phase (8 parts)	72:28	3.414	304621, 0.34	8450	1.07	3.67
		80:20	2.982	336521, 0.90	8254	1.08	5.01
		88:12	2.61	354890, 0.33	5378	1.05	3.83
Impurity-IV	Flow rate (mL/min.)	0.18	4.22	207416,0.84	5047	1.06	5.52
		0.20	5.464	25471846, 1.1	4972	1.02	6.35
		0.22	4.25	232601, 1.00	4818	1.06	5.51
	Mobile phase (8 parts)	72:28	4.38	197651, 0.52	5141	1.05	4.50
		80:20	5.464	25471846, 1.1	4972	1.02	6.35
		88:12	3.76	234988, 1.29	4750	1.06	5.76

*n = 3; RT-Retention time

Table 6: Results of forced degradation studies of Bictegravir

Condition	*RT (min)*	*Area count*	*USP plate count*	*USP resolution*	*USP tailing*	*% Degradation (%w/w)*	*Purity angle*	*Purity threshold*
Control	5.485	25465812	15838	-	1.05	0.00	5.329	12.475
Acid	5.475	21060147	15886	15.42	1.08	0.61	5.363	12.448
Alkali	5.477	22085976	15845	12.87	1.06	0.63	5.341	12.466
Oxidation	5.484	21563024	15877	18.63	1.07	0.73	5.368	12.452
Thermal	5.474	23085647	15861	9.78	1.09	0.42	5.375	12.443
Photolysis	5.484	25142478	15865	-	1.03	0.00	5.363	12.448

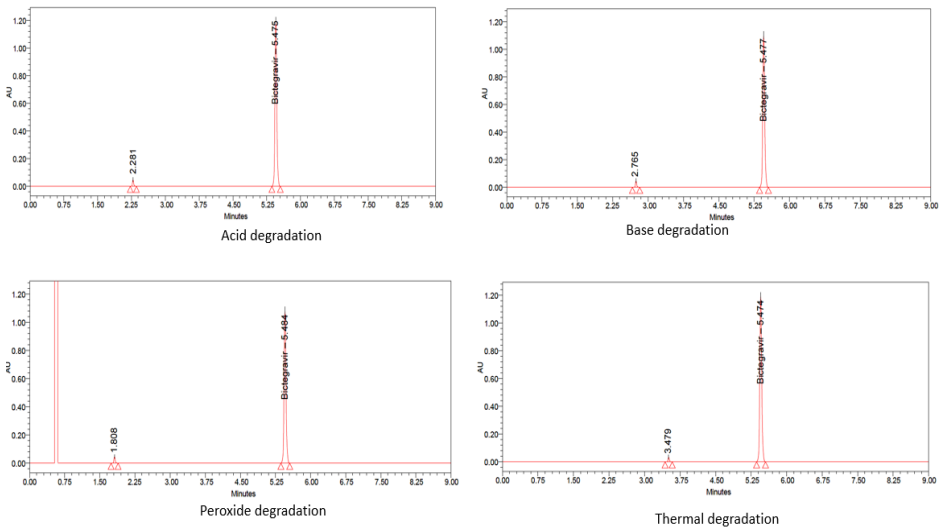

Figure 5: Forced degradation studies chromatograms

the peak threshold for degradants and BIC, representing the purity of the separated degradants. Significant % degradation was achieved in the case of acid, alkali, peroxide, thermal treatments, and minor in photolytic condition. The retention times were specific, and the chromatographic peak of BIC was not hindered in any manner due to the respective impurities spiked.

CONCLUSION

A precise, linear, fast, and LC method for Bictegravir and related compounds was developed and then validated satisfactorily in compliance with ICH Q2 (R2) standards. This newly developed LC technique was also utilized to confirm the stability of Bictegravir during storage and bulk specimens. Furthermore, the BIC was subjected to several stress conditions and tested using the UPLC technique. BIC was significantly destroyed by acidic, basic, and oxidative heat stress. However, it was shown to be stable under a photolytic environment. UPLC data was utilized to examine the deterioration products.

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