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DEVELOPMENT AND VALIDATION OF STABILITY-INDICATING UPLC METHOD FOR THE DETERMINATION OF BRIVARACETAM, ITS RELATED IMPURITIES AND DEGRADATION PRODUCTS

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Keywords:

Brivaracetam, Development, Forced degradation, UPLC, Validation, Stability indicating

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ABSTRACT: A novel stability-indicating mass compatible gradient Reverse Phase Ultra-Performance Liquid Chromatographic (RP-UPLC) method was developed for the quantitative determination of purity of brivaracetam drug substance samples in the presence of its impurities and degradation products. The method was developed using waters acquity UPLC BEH SHIELD RP18 (100 mm x 2.1 mm, 1.7 µm) column with mobile phase containing a gradient mixture of solvents A and B. The eluted compounds were monitored at 230 nm, the run time was 10 min within which brivaracetam and its four impurities were well separated. Brivaracetam was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. Brivaracetam was found to degrade significantly in acidic and slightly in oxidative stress conditions and stable in base, hydrolytic, and photolytic degradation conditions. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per ICH guidelines with respect to specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

INTRODUCTION: Brivaracetam (BCT) ((2S)-2-[(4R)-2-oxo-4-propylpyrrolidin-1-yl]) (**Fig. 1**), a chemical analog of levetiracetam, is a racetam derivative with anticonvulsant (antiepileptic) properties [1 - 3]. Brivaracetam is used to treat partial-onset seizures with or without secondary generalisation, in combination with other antiepileptic drugs. No data are available for its effectiveness and safety in patients younger than 16 years [4, 5].

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The most common adverse effects include sleepiness, dizziness, nausea and vomiting. More rarely, coordination problems and changes in behaviour can occur [6]. Administration of brivaracetam with phenytoin may increase phenytoin levels. Co-administration of other antiseizure drugs are unlikely to affect brivaracetam exposure.

Brivaracetam provides no added therapeutic benefit when administered in conjunction with levetiracetam that acts on the same protein. Brivaracetam is believed to act by binding to the ubiquitous synaptic vesicle glycoprotein 2A (SV2A), like levetiracetam but with 20-fold greater affinity [7]. There is some evidence that racetams including levetiracetam and brivaracetam access the luminal

side of recycling synaptic vesicles during vesicular endocytosis. They may reduce excitatory neuro-transmitter release and enhance synaptic depression during trains of high-frequency activity, such as is believed to occur during epileptic activity [8]. Brivaracetam exhibits linear pharmacokinetics over a wide dose range, is rapidly and completely absorbed after oral administration, has an elimination half-life of 7 to 8 hours, and has plasma protein binding of less than 20 % [9].

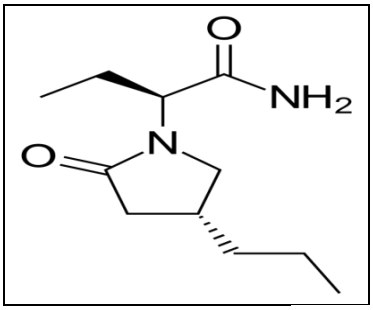

FIG. 1: CHEMICAL STRUCTURE OF BRIVARACETAM

N.V Mali and D. V. Mhaske have studied brivaracetam forced degradation analysis by HPLC [10]. However, neither the extent of degradation nor the high sensitivity of the method was reported. C. Alekhya *et al*., have been reported a review article on Brivaracetam drug action in human body [11]. So far, no study has been reported on the systematic analysis with high sensitive and precise method for degradation products of BCT under stress conditions prescribed by ICH Q1A (R2) [12].

To the best of our knowledge, none of the currently available analytical methods can separate and quantify all the known related compounds and degradation impurities of brivaracetam API. Furthermore, there is no stability-indicating HPLC/ UPLC method that was reported in the literature that can adequately separate and accurately quantify brivaracetam API. It is, therefore, felt necessary to develop a new stability indicating method for the related substances determination of brivaracetam.

We intend to opt for a faster chromatographic technique UPLC (Ultra high Performance Liquid Chromatography), for the said study and analytical validation [14] of developed method. An attempt has been made to determine whether UPLC can reduce analysis times without compromising the resolution and sensitivity. Hence a reproducible stability-indicating [15] RP-UPLC/PDA method was developed for the quantitative determination of Brivaracetam and its four impurities namely Imp - A, B, C and D.

Chemicals and Reagents: Brivaracetam (BCT) (99% purity) was a gift sample from a local manufacturing unit in Hyderabad, India. UPLC grade acetonitrile was purchased from Rankem (Mumbai, India). Analytical reagent grade sodium hydroxides, hydrochloric acid, hydrogen peroxide, 2, 2'-azobisisobutyronitrile (AIBN), ammonium acetate and trifluoro acetic acid were purchased from S.D. Fine Chemicals (Mumbai, India). Glass-distilled and deionized water (Nanopure, Bransted, USA) was used.

Instrumentation: For method development and forced degradation, studies were carried out on waters acquity UPLC system equipped with 2996 photodiode array detector. The output signal was monitored and processed by Empower software. For analysis of forced degradation samples to identify *m/z* values of degradants, Agilent 1200 series liquid chromatography coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrophotometer with Analyst 1.4 software, MDS, SCIEX, USA was used.

Preparation of solutions:

Preparation of Impurity Standard Solution: Impurity stock solution was prepared by dissolving appropriate amount of all the known impurities *i.e*. Imp-A, Imp-B, Imp-C and Imp-D in diluent (Acetonitrile and Solvent A in the ratio of 10:90, v/v) to get the final concentration of each impurity in stock solution as 20 µg mL^{-1}. As per ICH Q3A guidelines, specification limit of each known impurity in drug substance should not be more than 0.15 %, where the maximum daily dosage of drug is less than or equal to 2.0 g [12- 13]. The maximum daily dosage of BCT is less than 2.0g. Hence, considered specification limit for all the known impurities is 0.15 % and prepared impurity blend solution of 0.15 % by spiking appropriate volume of impurity stock solution in µL to 200 µg mL^{-1} BCT drug substance test solution.

Preparation of BCT Test Solution: BCT drug substance stock solution was prepared by weighing 20 mg of drug substance in 100 mL volumetric flask dissolved and diluted to volume with diluent. From this solution, 1 mL was transferred into 100 mL volumetric flask and diluted upto mark. 1mL of

this solution was further diluted to 10 with diluent. Final concentration of the solution was 0.2 µg mL^{-1} of BCT drug substance, which was used for related substances estimation.

Preparation of Forced Degradation Samples: As per ICH guidelines to generate degradation samples, one lot of BCT drug substance was selected and subjected to different stress conditions like acid hydrolysis, base hydrolysis, water hydrolysis, oxidation, photo degradation and Thermal degradation.

Preparation of Photo Degradation Sample: For photo degradation study, BCT drug substance was taken in petri dish and kept in UV cabinet. Both lamps of Ultra Violet and Visible radiations were kept in "ON" mode to expose the sample to both 254 nm and 365 nm. After 10 days, sample was taken -off from UV cabinet and test solutions were prepared to get the final concentration of 200 µg mL^{-1}.

Preparation of Thermal Degradation Sample: BCT drug substance was placed in petri dish and spread uniformly. The petri dish was kept in an oven and temperature of the oven was maintained at 60 °C for 10 days. After 10 days, BCT samples were taken-off from oven and the test solutions were prepared to get the final concentration of 200 µg mL^{-1}.

Preparation of Oxidative Degradation Sample: 200 mg of BCT drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of acetonitrile and made upto volume with 3 % aqueous peroxide solution. Placed magnetic stirrer in the solution and kept it at 60 °C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

Preparation of Water Hydrolysis Sample: 200 mg of BCT drug substance was transferred into a 100 mL volumetric flask, and dissolved in 10 mL of acetonitrile and made upto the volume with water. Placed magnetic stirrer in the solution and kept solution at 60°C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

Preparation of Acid Hydrolysis Sample: 200 mg of BCT drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of acetonitrile and made upto mark with 1N hydrochloric acid. Placed magnetic stirrer in the solution and kept solution at 60 °C temperature under continuous stirring for 4 h.

In order to prepare test solution for related substances estimation transferred 1 mL of above stressed sample solution into 10 mL volumetric flak, neutralized with 1N sodium hydroxide and made upto mark with diluent.

Preparation of Base Hydrolysis Sample: 200 mg of BCT drug substance was transferred to 100 mL volumetric flask, and dissolved in 10 mL of acetonitrile and made upto mark with 0.5N Sodium hydroxide solution. Placed magnetic stirrer in the solution and kept solution at 60 °C temperature under continuous stirring for 24 h. Diluted 1 mL of above stressed solution to 10 mL for the testing of related substances.

Sequential Steps in Method Development and Optimization: During initial development, impurity blend solution containing four potential impurities, Imp-A, Imp-B, Imp-C and Imp-D at 2 µg/mL spiked to 200 µg/mL of BCT drug substance was used. To confirm stability indicating power of the method, forced degradation samples were also considered for development.

Selection of Diluent: All the known impurities and drug substances were freely soluble in organic solvent, acetonitrile at 1 mg mL^{-1} concentration level. Hence, initial development trials were made with acetonitrile as a diluent. To get the gaussian peak shape for Imp-A under optimization of chromatographic conditions, the sample is dissolved in 1.0 mL of acetonitrile and made upto the volume with the solvent-A.

Selection of Wavelength: Injected brivaracetam, Imp-A, Imp-B, Imp-C and Imp-D in to the UPLC system and extracted the U.V spectra. U.V spectra of brivaracetam, Imp-A, Imp-B, Imp-C and Imp-D were presented as **Fig. 2**. BCT and its compounds showing wavelength maximum around 230 nm, hence it has been selected as UV detector wavelength for LC method development.

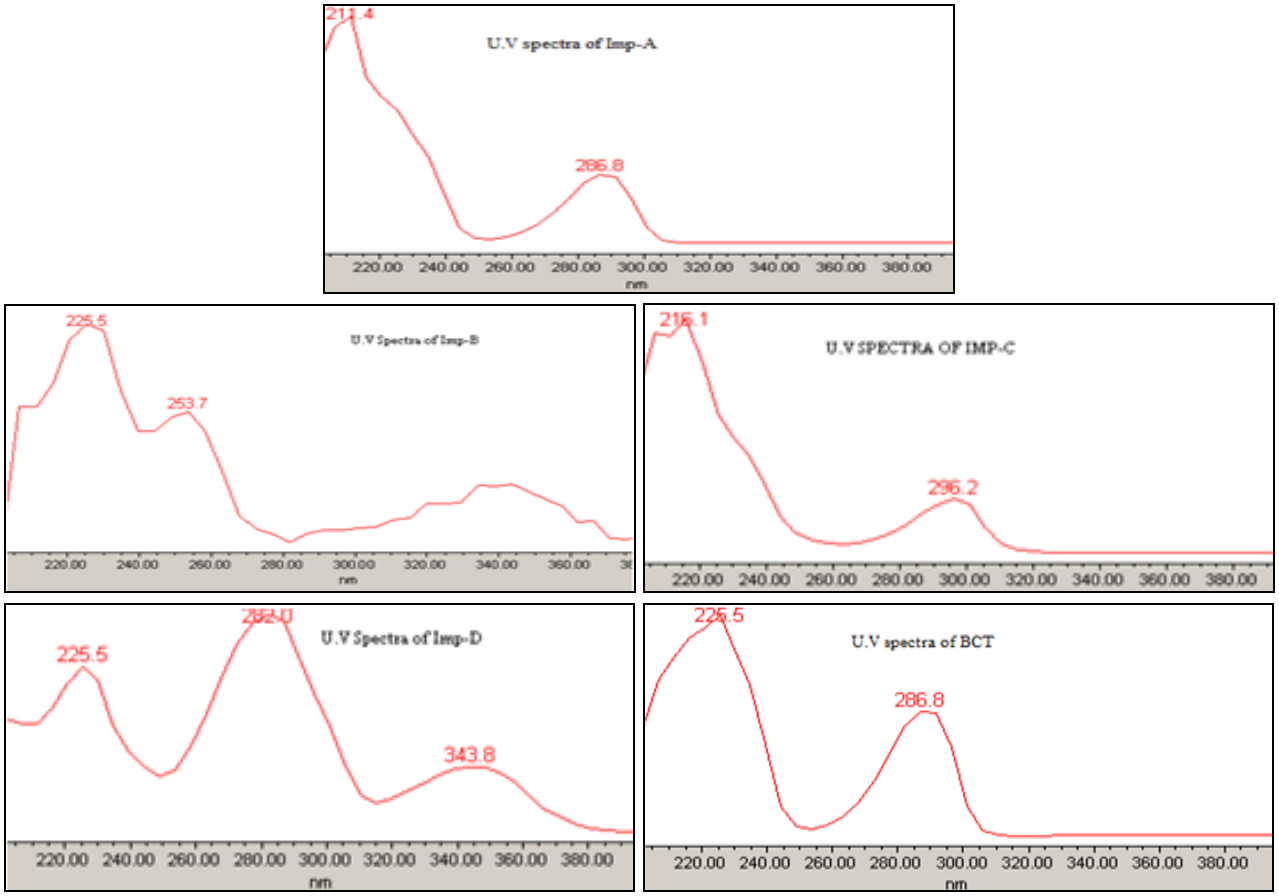

FIG. 2: TYPICAL UV SPECTRA OF BCT AND ITS RELATED IMPURITIES 8853186164

Method Development and Optimization: From the literature it was found that the pKa of the molecule is -0.84. Due to lower pKa of this molecule it was decided to adopt 0.1% Trifluoroacetic acid in water as solvent A. The blend containing 200µg/mL of brivaracetam and 2 µg/mL of each impurity (four) was prepared in the mixture of acetonitrile and solvent A (1:9, v/v). Brivaracetam spiked solutions were subjected to separation by reverse-phase LC on a waters acquity BEH C18, 50 x 2.1 mm, 1.7 µm column with 0.1% Trifluoroacetic acid in water as solvent A and acetonitrile, water (80:20, v/v) as solvent B. Flow rate was set at 0.3 mL/min. The UPLC gradient program (Time/% B) was set as 0.01/40, 8.0/90, 9.0/90, 9.01/40 and 10.0/40. Column temperature was maintained at 35°C (Trial-1). In this trial one of the unknown impurity closely eluting with Imp-A and other unknown impurity with Imp-C (Resolution<1.5) hence efforts were made to separate these closely eluting pair of compounds.

In order to increase the resolution between these pairs of compounds buffer composition was increased from 60 to 90 in the initial gradient step. With this increased buffer composition the retention time of brivaracetam was increased but Imp-A and its adjacent peak was co-eluting. Efforts were made to separate the pairs of compounds on waters acquity BEH C18, 100 x 2.1 mm, 1.7µm column. The chromatographic conditions of Trial-1 were employed in this trial. With the increase in column length Imp-A and its adjacent peak were separated (Resolution >2) but the resolution between Imp-C and its adjacent peak was not improved. Various trials were made by changing the gradient compositions but none of the trial could serve the purpose. It was decided to change the column chemistry and acquity UPLC BEH Shield RP18100 mm, 2.1 mm, and 1.7µm column was used with the conditions mentioned in trial-1.

It was found that all the peaks were separated with a resolution greater than 2. System suitability parameters were evaluated for brivaracetam and its four impurities. Tailing factor for all four impurities and brivaracetam was found less than 1.2. USP Resolution of brivaracetam and four potential impurities was greater than 2.0 for all pairs of compounds.

Optimized Chromatographic Conditions: Optimized chromatographic conditions for related substances estimation in BCT and quantification of BCT in drug substance was given in **Table 1**.

The retention times (RTs) and relative retention times (RRTs) of all the known compounds are presented in **Table 2**.

TABLE 1: FINAL CHROMATOGRAPHIC CONDITIONS OF BCT METHOD

Column	Waters Acquity UPLC BEH shield RP18, 100mm × 2.1mm, 1.7µparticle size.
Buffer	0.1 % of Trifluroroacetic acid in Milli-Q water
Mobile phase	Solvent-A : Degassed buffer Solvent-B : Water : Acetonitrile : 20: 80 (v/v)
Mode of elution	Gradient
Flow rate	0.3 mL min^{-1}
Column temperature	35 °C
Wavelength of detection	230 nm
Injection volume	1.0 µL
Run time	10 min
Diluent	Acetonitrile and Solvent-A in the ratio 1:9 (v/v)
Gradient program	Time (min)/ % Mobile phase- B: 0.01/40, 8.0/90, 9.0/90, 9.01/40 and 10.0/40.
Concentration	For related substances estimation : 200 µg mL^{-1}

TABLE 2: RTS AND RRTS OF KNOWN COMPONENTS IN FINALIZED METHOD

S. no.	Name of the Analyte	Retention time (min)	Relative retention time w.r.t. BCT peak
1	Imp-A	1.204	0.38
2	Imp-B	2.546	0.81
3	Imp-C	4.274	1.36
4	Imp-D	4.461	1.42
5	BCT	3.134	1.00

Discussion on Forced Degradation Studies: Analyzed BCT drug substances were spiked with all the known impurities, Imp-A, Imp-B, Imp-C and Imp-D at 0.15% level with respect to test concentration.

The spectral homogeneity of main component was ensured in spiked solution. Spiked sample chromatogram with all known impurities and peak purity plot for BCT peak in spiked solution is presented in **Fig. 3** and **4**.

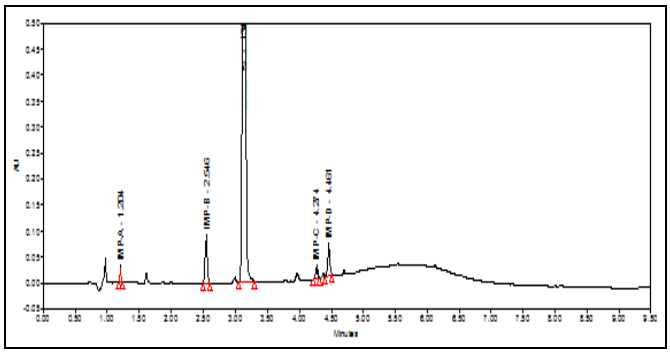

FIG. 3: CHROMATOGRAM OF BCT SPIKED WITH ALL KNOWN COMPONENTS

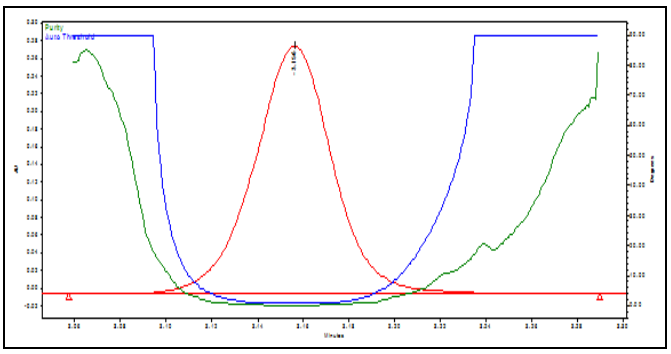

FIG. 4: PEAK PURITY PLOT FOR BCT PEAK IN SPIKED SOLUTION

All the forced degradation samples were analyzed in UPLC method by using PDA detector. Homogeneity of the BCT peak is confirmed in all the degradation samples.

Degradation of BCT during Oxidation: BCT drug substance was sensitive towards the oxidation, it undergone degradation and formed impurity at RRT 1.26 and 1.56. Oxidative degradation conditions and time are discussed in preparation of oxidative degradation sample section. Peak purity of BCT peak in oxidized drug substances was confirmed as homogeneous. Chromatogram of BCT sample after oxidation and peak purity plot of BCT peak are presented in **Fig. 5** and **6**.

Degradation during Base Hydrolysis: BCT drug substance was subjected to base hydrolysis by using 0.5N Sodium hydroxide at 60°C temperature. Hydrolyzed sample solution was analyzed and monitored upto 24 h. No significant degradation was observed in basic conditions. Based on peak

purity data it was confirmed that the main component in base hydrolyzed sample is homogeneous. Chromatogram of base hydrolyzed BCT sample and purity plots are presented in **Fig.7** and **Fig. 8** respectively.

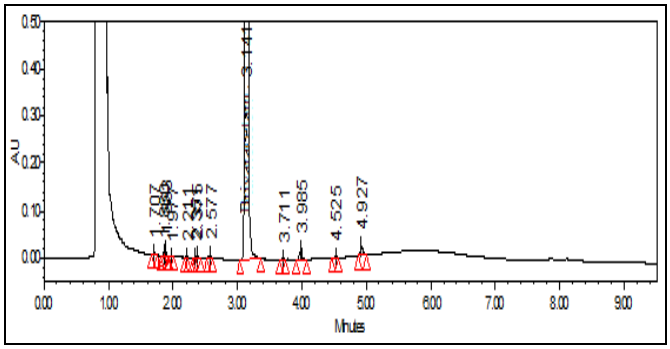

FIG. 5: CHROMATOGRAM OF OXIDIZED BCT SAMPLE

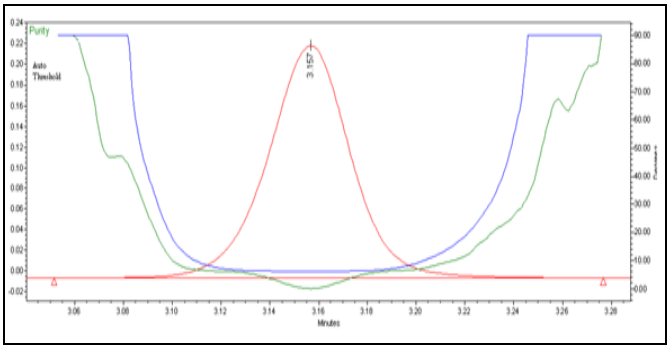

FIG. 6: PEAK PURITY PLOT FOR BCT PEAK IN OXIDIZED SAMPLE

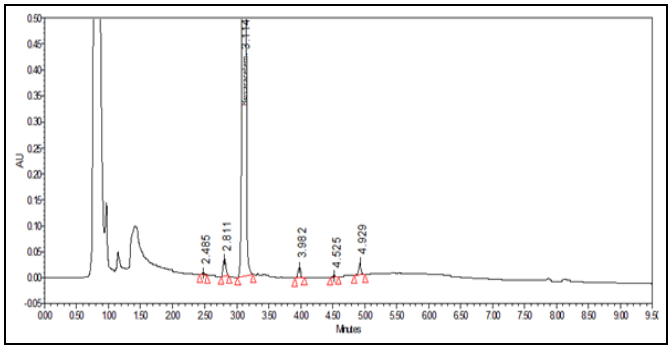

FIG. 7: CHROMATOGRAM OF BASE HYDROLYSIS BCT SAMPLE

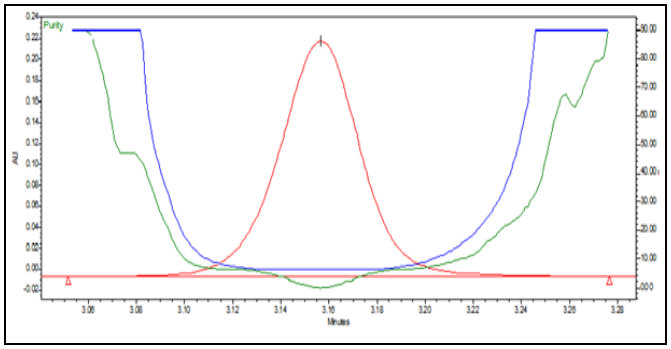

FIG. 8: PEAK PURITY PLOT FOR BCT PEAK IN BASE HYDROLYSIS

Degradation during Acid Hydrolysis: When, BCT drug substance was subjected to acid hydrolysis by using 1N Hydrochloric acid at 60 °C, significant degradation was observed. After 4h of acid hydrolysis, around 12.6 % of BCT was getting degraded leading to a major peak at 0.38 RRT. Peak purity of BCT peak in acid hydrolyzed sample was found to be homogeneous. LC chromatogram of acid hydrolyzed sample and peak purity plots are shown in **Fig. 9** and **Fig. 10** respectively.

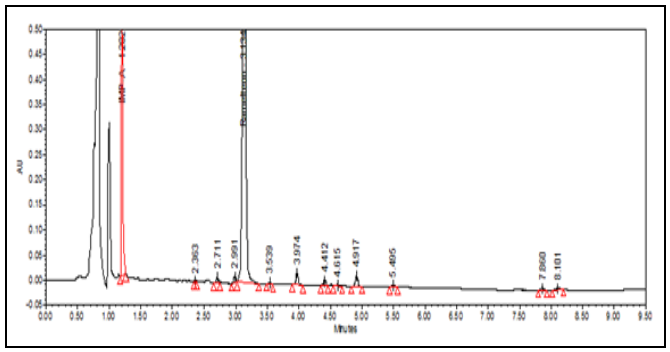

FIG. 9: CHROMATOGRAM OF ACID HYDROLYSIS BCT SAMPLE ACID

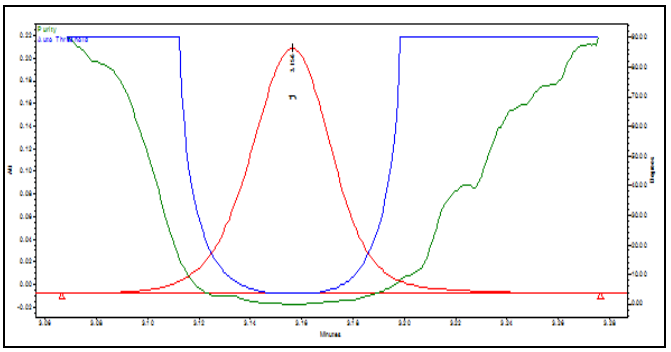

FIG. 10: PEAK PURITY PLOT FOR BCT PEAK IN HYDROLYSIS

Impurity at RRT 0.38 was identified by LC-MS analysis. It exhibited molecular ion at m/z 212.14 amu $(M+H)^+$ and corresponds to Imp-A. LC-MS analytical conditions are described in below section. Mass spectral data of acid degradation is presented.

LC-MS Analytical Conditions: LC–MS/MS system (Agilent 1200 series liquid chromatography coupled with Applied Biosystems 4000 Q Trap triple quadruple mass spectrometer with Analyst 1.4 software, MDS SCIEX,USA) was used for the generation of mass spectral data of unknown

compounds formed during forced degradation studies. Develosil ODS MG-5, 250 x 4.6 mm, 5µm column (Nomura Chemical Co, Japan) was used as stationary phase. 0.1% trifluoroacetic acid (ACROS ORGANICS, Geel, Belgium) was used as buffer. 100 % Buffer was used as solvent A and buffer and acetonitrile in the ratio 15:85, v/v; was used as solvent B. The gradient program (T/%B) was set as 0.01/35, 20/70, 40/80, 45/95, 64/95, 65/35 and 70/35. Mixture of acetonitrile and Solvent A in the ration 1:9, v/v; was used as diluent. The flow rate was 1.0 ml/min.

The analysis was performed in positive electro spray positive ionization mode. Ion Source voltage was 5000V. Source temperature was 450 °C. GS1 and GS2 are optimized to 30 and 35 psi respectively. Curtain gas flow was 20 psi. To further confirm that the acid hydrolysis peak is Imp-A, spiked Imp-A to the acid hydrolysis sample and verified the peak purity. The peak was found to be spectrally pure, confirming that the acid hydrolysis of BCT leads to the formation of Imp-A. Peak purity plot of acid hydrolysis degradant is shown in **Fig. 12**.

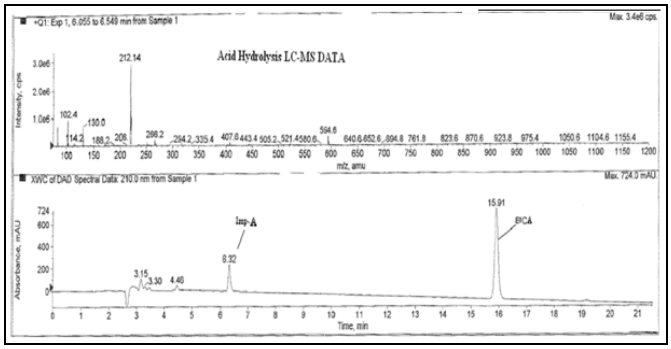

FIG. 11: MASS SPECTRA OF DEGRADANT FOUND DURING ACID HYDROLYSIS

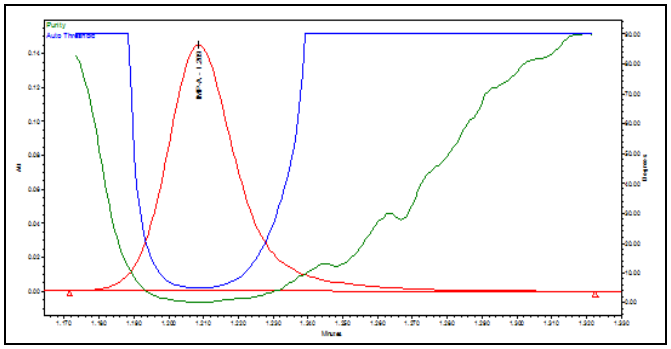

FIG. 12: PEAK PURITY PLOT FOR ACID HYDROLYSIS PEAK

Degradation during Water Hydrolysis: BCT drug substance is more stable towards water hydrolysis; BCT was not degraded even after 24 h of water hydrolysis at 60 °C. Peak purity data of BCT peak in final sample indicates that peak is homogeneous. Chromatogram of stressed sample and peak purity plot of BCT are shown in **Fig. 13** and **Fig. 14** respectively.

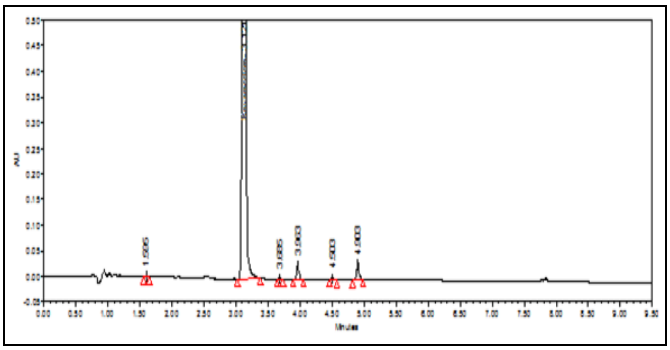

FIG. 13: CHROMATOGRAM OF WATER HYDROLYSIS BCT SAMPLE

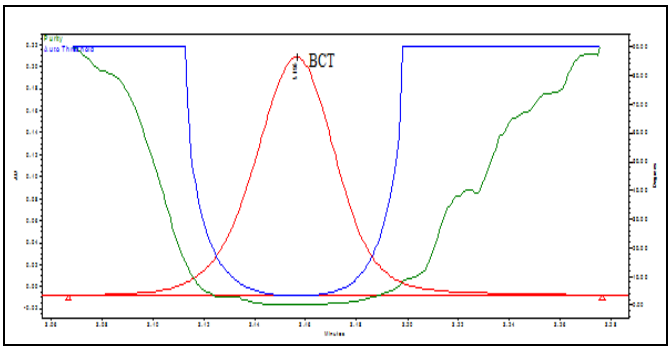

FIG. 14: PEAK PURITY PLOT FOR BCT PEAK IN WATER HYDROLYSIS

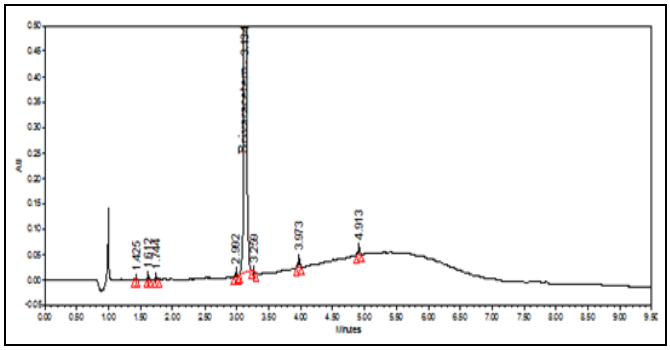

FIG. 15: CHROMATOGRAM OF THERMAL DEGRADATION BCT SAMPLE

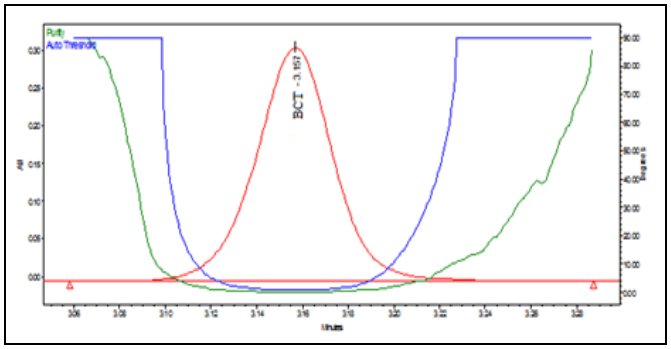

FIG: 16. PEAK PURITY PLOT FOR BCT PEAK IN THERMAL DEGRADATION

Thermal Degradation BCT drug substance was thermally stable. The drug was exposed to 60 °C temperature for 10 days, practically it was not degraded. BCT peak in thermally degraded sample was found to be spectrally pure. Chromatogram of stressed sample and peak purity plot is shown in **Fig. 15** and **Fig. 16**.

Photolytic Degradation:

Results of Forced Degradation Studies: BCT drug substance was exposed to UV and visible radiations for 10 days. No considerable degradation was observed. To confirm the stability of BCT drug substance towards photolytic condition, peak purity of BCT peak was verified in degraded sample and found that it was homogeneous. Chromatogram of photolytic degradation sample and peak purity plot shown in **Fig. 17** and **18** respectively. The summary of force degradation studies were reported in **Table 3**.

Analytical Method Validation: The developed and optimized LC method was fully validated as per ICH and USP guidelines.

System Suitability Test (SST): Prepared six different test solutions of BCT drug substance, they were spiked with all specified known impurities, Imp-A, Imp-B, Imp-C and Imp-D at 0.15 % level with respect to test concentration. These solutions were injected into LC system. System suitability results were tabulated in **Table 4**. System suitability chromatogram is shown in **Fig. 19**.

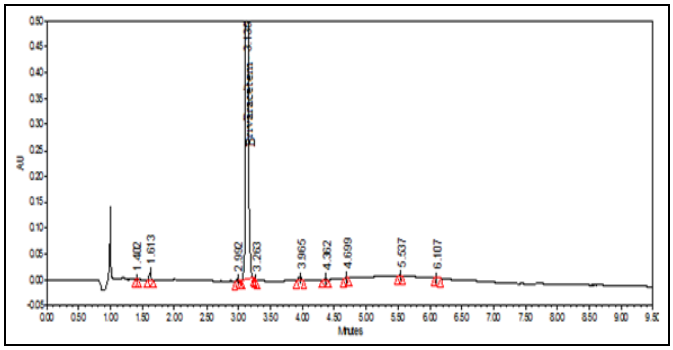

FIG. 17: CHROMATOGRAM OF PHOTOLYTIC DEGRADATION BCT SAMPLE

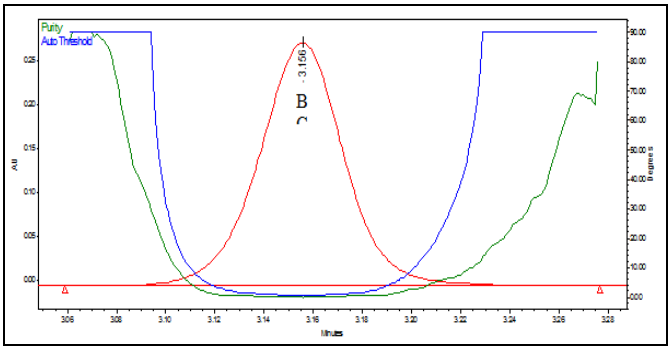

FIG. 18: PEAK PURITY PLOT FOR BCT PEAK IN PHOTOLYTIC DEGRADATION SAMPLE

TABLE 3: RESULTS OF FORCED DEGRADATION STUDIES

Degradation condition	Time	RS by UPLC % of degradation	Remarks/observation
HCl- 1N 60 °C (Acid hydrolysis)	4 hr	12.6 %	Impurity-A formed
NaOH-0.5N 60 °C (Base hydrolysis)	24 hrs	0.3 %	No significant degradation observed
Water hydrolysis (60 °C)	24 hrs	0.2 %	No significant degradation observed
Oxidation by H_2O_2- 3.0% 60 °C	24 hrs	1.5 %	No significant degradation observed
Thermal (60 °C) solid	10 days	0.2 %	No degradation observed
UV at 254nm & 365nm	10 days	0.2 %	No degradation observed

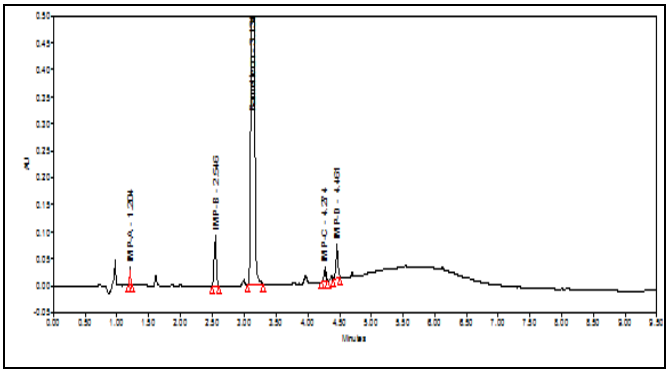

FIG. 19: SYSTEM SUITABILITY CHROMATOGRAM

Precision: Precision study has been evaluated by performing both repeatability and intermediate precision. To ensure the repeatability of related substances method, six individual preparations of BCT drug substance was prepared and spiked with Imp-A, Imp-B, Imp-C and Imp-D at specification level (0.15%) with respect to test concentration.

TABLE 4: SST RESULTS FOR BCT RELATED SUBSTANCES

Name of the Compound	Retention time (min)	RRT w.r.t. BCT peak
Imp-A	1.204	0.38
Imp-B	2.546	0.81
Imp-C	4.274	1.36
Imp-D	4.461	1.42
BCT	3.134	1.00

Later calculated the % RSD for each individual known impurity content. Results are tabulated in **Table 5**. The above results are evidently show that the method is repeatable within acceptable limits of % RSD for six preparations of related substances; 0.81-3.90.

TABLE 5: RESULTS OF BCT RELATED SUBSTANCES METHOD PRECISION

Sample solution	% of Related substances			
	Imp-A	Imp-B	Imp-C	Imp-D
Preparation-1	0.14	0.16	0.14	0.15
Preparation-2	0.13	0.16	0.14	0.15
Preparation-3	0.13	0.16	0.15	0.14
Preparation-4	0.13	0.16	0.14	0.15
Preparation-5	0.14	0.15	0.14	0.15
Preparation-6	0.14	0.16	0.14	0.15
Mean	0.14	0.16	0.14	0.15
% RSD	3.90	0.87	1.41	0.81

Intermediate precision or Ruggedness of BCT RS method was demonstrated by performing precision study as mentioned in repeatability testing on two different days, by a different analyst, using different lots of reagents, different column and by using different equipment. Then the % RSD for content of each impurity was calculated. Ruggedness of BCT RS method is tabulated in **Table 6**. Results have shown insignificant variation in measured response in two different days, which demonstrated that the method was highly precise for its intended use of estimation of related substances of BCT.

TABLE 6: RESULTS OF RUGGEDNESS FOR BCT RS METHOD

Name of Analyte	% RSD for six different preparation	
	Day-1	Day-2
Imp-A	3.90	1.57
Imp-B	0.87	0.77
Imp-C	1.41	1.10
Imp-D	0.81	1.06

Sensitivity: Sensitivity of the method was demonstrated in terms of Limit of Quantitation (LOQ) and Limit of Detection (LOD) values of specified analytes. LOQ, LOD values were established for Imp-A, Imp-B, Imp-C, Imp-D and BCT based on signal to noise ratio of each peak.

Limit of Quantification (LOQ): Prepared a series of dilutions of BCT, Imp-A, Imp-B, Imp-C and Imp-D in different concentrations and injected them into the liquid chromatography to get the signal to noise ratio 10 (*i.e*. 9.5 - 10.0). Limit of quantitation values of all the analytes are presented in **Table 7**.

TABLE 7: LOQ VALUES BCT AND ITS RELATED COMPOUNDS

S. no.	Name of the Analyte	LOQ in µg/mL	% w.r.t. test concentration
1	Imp-A	0.07	0.035
2	Imp-B	0.04	0.020
3	Imp-C	0.08	0.040
4	Imp-D	0.06	0.030
5	BCT	0.05	0.025

Limit of Detection (LOD): Prepared a series of dilutions of BCT, Imp-A, Imp-B, Imp-C and Imp-D in different concentrations and injected them into the liquid chromatography to get the signal to noise ratio 2 - 3. Limit of detection values of all the analytes are presented in **Table 8**.

TABLE 8: LOD VALUES OF THE IMPURITIES AND BCT PEAK

S. no.	Name of the analyte	LOD in µg/mL	% w.r.t. test concentration
1	Imp-A	0.022	0.011
2	Imp-B	0.014	0.007
3	Imp-C	0.025	0.013
4	Imp-D	0.021	0.011
5	BCT	0.018	0.009

Precision at Limit of Quantification Level: Six preparations of Imp-A, Imp-B, Imp-C and Imp-D at LOQ level were injected individually and the % RSD for the areas of each analyte was calculated. No significant variation was observed in the area of each analyte for six consecutive injections, %RSD of all the components ranges from 0.8 to 8.3 %. Results are summarized in **Table 9**.

TABLE 9: LOQ PRECISION RESULTS OF BCT RELATED COMPOUNDS

Preparation	Area of Imp-A	Area of Imp-B	Area of Imp-C	Area of Imp-D
Preparation-1	756	3355	1116	2848
Preparation-2	712	3347	1127	2801
Preparation-3	754	3225	1158	2853
Preparation-4	604	3339	1169	2824
Preparation-5	666	3431	1179	2868
Preparation-6	725	3372	1145	2853
Mean	702.8	3344.8	1149	2841
Stdev.	58.6	67.3	24.4	24.3
% RSD	8.3	2.0	2.1	0.8

Above results indicate that BCT RS method is precise at LOQ level

Accuracy at LOQ Level: BCT sample was injected in test concentration *i.e*. 200 µg mL^{-1} to estimate the content of Imp-A, Imp-B, Imp-C and Imp-D. Three different sample solutions (200 µg mL^{-1}) of BCT containing Imp-A, Imp-B, Imp-C and Imp-D at LOQ level were prepared and

injected each solution once. From the corrected area of Imp-A, Imp-B, Imp-C and Imp-D, % recovery of each impurity was calculated. Results are summarized in **Table 10**.

TABLE 10: RESULTS OF ACCURACY AT LOQ LEVEL

Name	Workup	Amount added (µg/mL)	Amount obtained (µg/mL)	% Recovery	% Mean recovery
Imp-A	1	0.0672	0.0636	94.6	105.2
	2		0.0736	109.5	
	3		0.0749	111.5	
Imp-B	1	0.0413	0.0429	104.0	103.3
	2		0.0425	103.0	
	3		0.0425	103.0	
Imp-C	1	0.0758	0.0693	91.4	99.3
	2		0.0792	104.4	
	3		0.0774	102.2	
Imp-D	1	0.0630	0.0658	103.9	104.4
	2		0.0649	104.2	
	3		0.0666	105.1	

Linearity A series of linearity solutions were prepared containing BCT, Imp-A, Imp-B, Imp-C and Imp-D solution at different concentrations *i.e.* 0.038 %, 0.075 %, 0.125 %, 0.15 %, 0.19 % and 0.225 % of working concentration (200 µg mL^{-1}) by performing appropriate dilutions to achieve the targeted concentrations. The above prepared solutions of BCT and its impurities are 25%, 50%, 100%, 125% and 150% to known impurity specification limit *i.e.* 0.15 %. Each solution was injected once and calibration plots were drawn for concentration of each component versus peak area of corresponding known component.

Linearity plot of each analyte with best fit linear equation is shown in **Fig. 20 - 24**. Linear regression analysis was performed for each analyte and data is presented in **Table 11-15**.

TABLE 11: LINEARITY OF IMP-A

S. no.	Conc. (µg mL^{-1})	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.075	2902	2953	51	2572	38693
2	0.150	5818	5981	163	26644	38787
3	0.225	9460	9010	-450	202731	42044
4	0.300	11794	12038	244	59662	39313
5	0.375	15111	15067	-44	1956	40296
6	0.450	18059	18095	36	1317	40131
Regression coefficient		0.9991	Residual sum of squares		294881	
Slope		40380	Intercept		-76	
% y-Intercept		-0.64	Linearity equation		$y = 40380x - 76$	

TABLE 12: LINEARITY OF IMP-B

S. no.	Conc. (µg mL^{-1})	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.08625	18076	18494	418	175003	209577
2	0.1725	37826	37431	-395	156394	219281
3	0.25875	56962	56367	-595	354342	220143
4	0.345	74148	75303	1155	1333871	214922
5	0.43125	95273	94239	-1034	1068880	220923
6	0.5175	112724	113175	451	203702	217824
Regression coefficient		0.9997	Residual sum of squares		3292192	
Slope		219550				
Intercept		-442				
% y-Intercept		-0.60	Linearity equation		$y = 219550x - 442$	

TABLE 13: LINEARITY OF IMP-C

S. no.	Conc. (µg mL^{-1})	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.084375	7024	6293	93	8693	73481
2	0.16875	12019	11968	-51	2641	71224
3	0.253125	18102	17642	-460	211618	71514
4	0.3375	23050	23316	266	70944	68296
5	0.421875	28404	28991	587	344245	67328
6	0.50625	35100	34665	-435	189142	69333
Regression coefficient		0.9993	Residual sum of squares		827282	
Slope		65857				
Intercept		1168				
% y-Intercept		2.68	Linearity equation		$y = 65857x + 1168$	

TABLE 14: LINEARITY OF IMP-D

S. no.	Conc. (µg mL-1)	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.084375	11332	11964	632	399484	134305
2	0.16875	22625	22559	-66	4335	134074
3	0.253125	33770	33154	-616	379116	133412
4	0.3375	43851	43749	-102	10324	129929
5	0.421875	55191	54345	-846	716554	130823
6	0.50625	63942	64940	998	995244	126305
Regression coefficient		0.9994	Residual sum of squares		2505057	
Slope		125572				
Intercept		1369				
% y-Intercept		3.12	Linearity equation		$y = 125572x - 1369$	

TABLE 15: LINEARITY OF BCT FOR RS METHOD

S. no.	Conc. (µg mL-1)	Imp-A peak area	Calculated response through trend line equation	Residual	Residual square	Sensitivity
1	0.0675	2416	2662	246	60352	35793
2	0.135	5430	5140	-290	83830	40222
3	0.2025	7685	7619	-66	4321	37951
4	0.27	10065	10098	33	1093	37278
5	0.3375	12516	12577	61	3705	37084
6	0.405	15040	15056	16	245	37136
Regression coefficient		0.9993	Residual sum of squares		153546	
Slope		36723				
Intercept		183				
% y-Intercept		1.82	Linearity equation		$y = 36723x + 183$	

Regression coefficients for concentration against peak area of all the related compounds of BCT and BCT peak from 25% to 150 % level to the specification limit were more than 0.99. And also % of y-bias with respect to 100 % specification of known compounds is within the limit of ± 5.0. This indicates that a developed related substance by LC method for BCT was linear.

Accuracy: Solutions of Imp-A, Imp-B, Imp-C and Imp-D at three different concentration levels *i.e*. 50 %, 100 % and 150 % w.r.t. the specification limit (0.15%) of working concentration was spiked with BCT sample solution (conc. 200 µg mL^{-1}), each level was prepared in triplicate and each of nine solutions were injected once. Amount of Imp-A, Imp-B, Imp-C and Imp-D obtained in each solution was calculated as % recovery. Accuracy results at three levels are summarized in **Table 16**.

% recoveries obtained for three different levels ranged from 95.6 to 109. Stand deviation for the average of % recoveries of each individual analyte is less than 5.0. Above accuracy results reveal that this method is highly accurate.

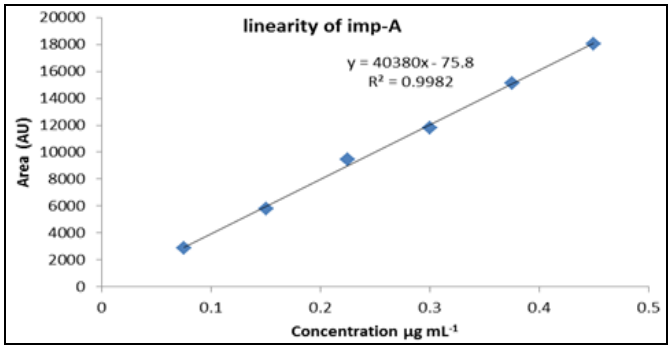

FIG. 20: LINEARITY PLOT FOR IMP-A

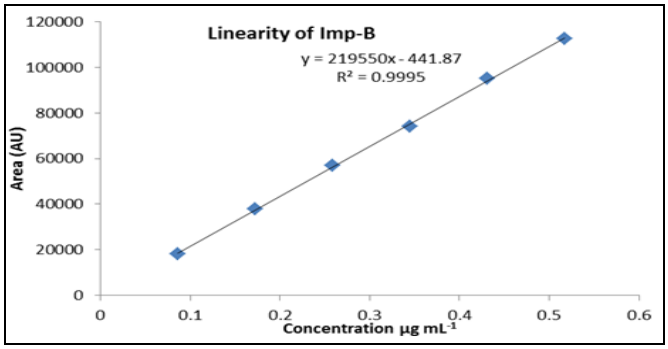

FIG. 21: LINEARITY PLOT FOR IMP-B

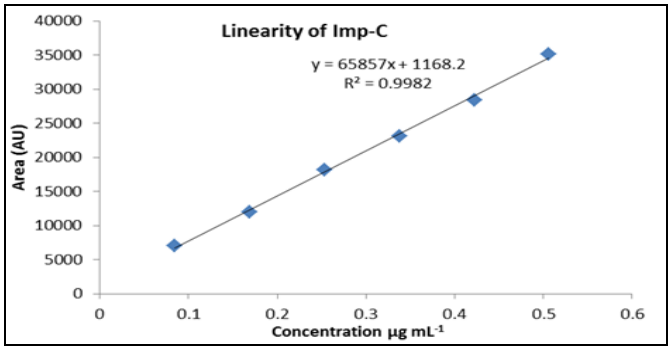

FIG. 22: LINEARITY PLOT FOR IMP-C

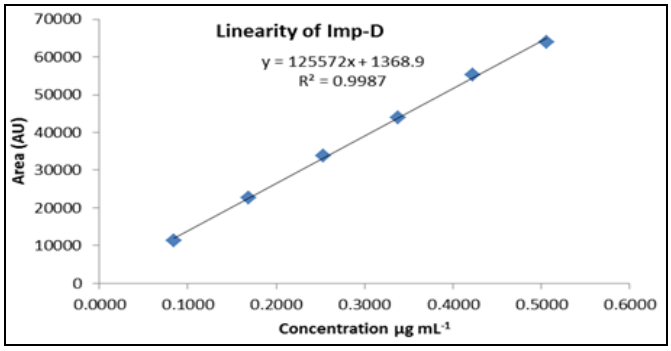

FIG. 23: LINEARITY PLOT FOR IMP-D

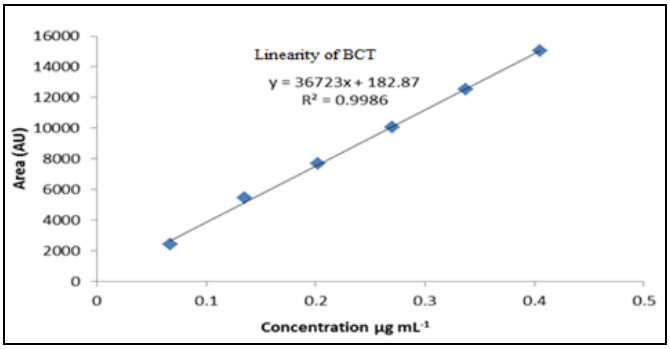

FIG. 24: LINEARITY PLOT FOR BCT FOR RS METHOD

TABLE 16: ACCURACY RESULTS

Name of the analyte	Concentration of analyte w.r.t. specification limit	Amount of impurity spiked to BCT* (µg mL-1)	Amount of impurity recovered (µg mL-1)	% Mean recovery ± SD
Imp-A	50 %	0.1500	0.1470	98.0 ± 4.6
	100 %	0.3000	0.2867	95.6 ± 3.7
	150 %	0.4500	0.4691	104.2 ± 0.2
Imp-B	50 %	0.1725	0.1768	102.5 ± 0.4
	100 %	0.3450	0.3533	102.4 ± 0.5
	150 %	0.5175	0.5361	103.6 ± 0.4
Imp-C	50 %	0.1688	0.1840	109.0 ± 3.7
	100 %	0.3375	0.3553	105.3±1.5
	150 %	0.5063	0.5278	104.2 ± 0.5
Imp-D	50 %	0.1688	0.1706	101.1 ± 1.0
	100 %	0.3375	0.3411	101.1 ± 0.9
	150 %	0.5063	0.5150	101.7 ± 1.2

Range: As evident from linearity, accuracy and precision study of related substances method, range has been established for all the analytes *i.e.* BCT, Imp-A, Imp-B, Imp-C and Imp-D from LOQ to 150 % of specification limit.

Robustness: To evaluate the influence of minute changes in finalized method parameters on separation of known components, robustness study was performed. Study was done by deliberately altering the method conditions from the original

method parameters and verified RRTs of impurities and system suitability parameters of standard solution. Method parameters selected for the study were, flow rate (± 0.03 mL/min) and column temperature (± 5 °C). Robustness study data of BCT related substances method is provided in **Table 17**. Above results of BCT RS method robustness study reveals that no significant variation was found in the SST results and RRTs of BCT related substances. Hence, the developed method has been considered as robust.

TABLE 17: ROBUSTNESS STUDY DATA

Parameter and variation	RRTs of impurities			
	Imp-A	**Imp-B**	**Imp-C**	**Imp-D**
As such conditions	0.38	0.81	1.36	1.42
Flow rate (mL min^{-1})				
a. 0.27	0.39	0.82	1.35	1.41
b. 0.33	0.38	0.80	1.38	1.44
Column Temperature (°C)				
a. 30	0.38	0.82	1.38	1.44
b. 40	0.38	0.81	1.35	1.41

Conclusion from Analytical Method Validations: The developed RP-LC method developed for quantitative determination of related substances of BCT in drug substance is precise, accurate, selective and linear as per the ICH recommended guidelines. The Robustness and ruggedness or intermediate precision study reveals that the method is highly rugged and robust for its intended use.

CONCLUSION: The proposed RP-UPLC method is sensitive, linear, precise and accurate for quantitation of related substances of BCT and its degradation products. As the method was fully validated as per ICH and proved the stability indicating power, it can be used for estimation of impurities in BCT for routine analysis, stability testing in Pharmaceutical quality control labs.

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