STABILITY INDICATING METHOD DEVELOPMENT AND VALIDATION FOR THE DETERMINATION OF CRISABOROLE IN BULK DRUGS BY USING UPLC

[1]Syed Mastan Ali, [2]D. Ramachandran

Department of Chemistry,
Acharya Nagarjuna University, Guntur-522510, Andhra Pradesh, India.

Corresponding author: Syed Mastan Ali

***Abstract*: The validated analytical method was applied for the estimation of Crisaborole(CRB) by reverse phase ultra-performance liquid chromatography (RP-UPLC). The drug was subjected to various stress Conditions such as hydrolysis, oxidation, photolytic and thermal degradations to investigate the stability indicating ability of the method. Compound is highly sensitive against hydrolytic stress conditions like basic and oxidative stress conditions. In oxidative condition product completely converted into degradation product with boron ring got opened. Efficient chromatographic separation was achieved by using Acquity; UPLC, CSH; C-18; 100 x 2.1mm; 1.7 µm column with the mobile phase consisting of 0.1% Trifluoro acetic acid in water and 0.1% Trifluoro acetic acid in acetonitrile in a gradient elution mode within a short run time of 6.0 minutes at a flow rate of 0.4 ml/min with column temperature at 25°C. The developed method was validated as per the current ICH quality guidelines with respect to specificity, precision, accuracy, linearity, robustness and solution suitability. The average recovery values of Crisaborole were found to be in the range of 100.05-101.16 %. The developed method was linear with the correlation value of 0.9995 for Crisaborole. The repeatability and intermediate precision expressed by RSD were less than 2.0% for Crisaborole. The test solution was found to be stable in diluent for 72 h when stored at room temperature. The developed UPLC method is superior in technology against conventional HPLC with respect to speed, resolution, solvent consumption and cost of analysis. This method is compatible to LCMS analysis which enables to identify the unknown impurities or the degradants formed in the process.**

***Keywords*: Crisaborole, stability indicating UPLC method, Assay validation.**

Introduction:

Crisaborole with chemical name as 4-[(1-hydroxy-1,3-dihydro-2,1-benzoxaborol-5-yl)oxy] benzonitrile) with chemical formula C14H10BNO3 and its mol. weight is 251.05 is a member of the class of benzoxaboroles characterized by the presence of a boronic acid hemiester with a phenolic ether and a nitrile. It is used as a topical treatment of psoriasis. Crisaborole sold under the brand name Eucrisa among others, is a nonsteroidal topical medication used for the treatment of mild-to-moderate atopic dermatitis (eczema) in adults and children. Crisaborole is a phosphodiesterase 4 (PDE-4) inhibitor. Crisaborole is a novel oxaborole approved by FDA as a topical treatment of for mild to moderate atopic dermatitis [1-2]. This non-steroidal agent is efficacious in improving disease severity, reducing the risk of infection, and reducing the signs and symptoms in patients 2 years old and older. It reduces the local inflammation in the skin and prevents further exacerbation of the disease with a good safety profile. Its structure contains a boron atom, which facilitates skin penetration and binding to the bimetal center of the phosphodiesterase 4 enzyme [3-4]. Crisaborole is a boron compound recently approved by the US Food and Drug Administration as a 2% ointment for the treatment of mild to moderate atopic dermatitis. In 2016 for the topical treatment of mild to moderate AD and up to now is the only formulation available on the market (Eucrisa, Pfizer). Although crisaborole has been extensively studied in vivo for the treatment of AD (Paller et al., 2016; Simpson et al., 2018; Zane et al., 2016; Zane, Hughes, & Shakib, 2016) and other skin conditions such as psoriasis (Nazarian & Weinberg, 2009; Robbins, Gor, & Bui, 2018), there is no current literature documenting in-vitro skin transport studies, except for the work of Jarnagin et al. (2016) [4-6]. In this paper, permeation and retention data of crisaborole across and in human cadaver skin using 5% ointment are described, but no details about extraction from the skin layers or the analytical methods are reported. Crisaborole is a phosphodiesterase-4 inhibitor, mainly acting on phosphodiesterase 4B (PDE4B), which causes inflammation. Chemically, crisaborole is a phenoxybenzoxaborole. Inhibition of PDE4B appears to suppress the release of tumor necrosis factor alpha (TNFα), interleukin-12 (IL-12), IL-23 and other cytokines, proteins believed to be involved in the immune response and inflammation. People with atopic dermatitis produce high levels of proteins called cytokines, which can cause the inflammation of the skin seen in dermatitis. Crisaborole blocks the release of certain cytokines involved in the inflammation process such as tumor necrosis factor alpha, interleukins (IL-2, IL-4, IL-5), and interferon gamma. By blocking their release, crisaborole is expected to ease the inflammation and therefore relieve symptoms of the disease [7-9]. Hence there exists a need for development of quick and simple separation method for purity control of Crisaborole in bulk and formulations. The purpose of current research was to develop a method for stability indicating assay quantification method for CRB by using ultra performance liquid chromatography (UPLC) [10-14]. This technique has the minimal drawbacks which arise from high column back-pressure when compared to the same separations Obtained with traditional HPLC instrument. With minimal void volumes and maximal sensitivity, faster separations can be achieved with higher efficiencies. It provides apt resolution with sharp peak shapes using low particle size columns and also detects the low level of force degradation impurities providing ready access for validation using compliance software. The developed method was novel and unreported in literature.

Structure of Crisaborole:

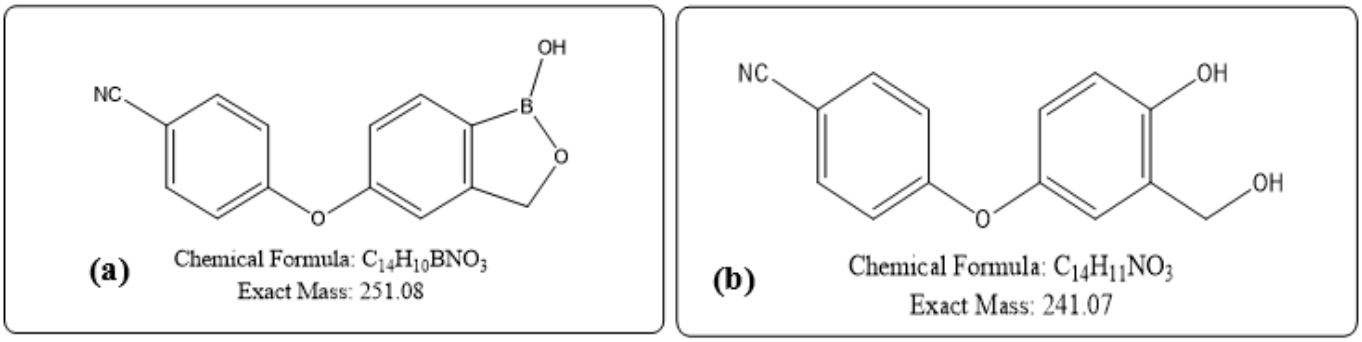

Fig.1: a). Structure of Crisaborole and **b).** Possible degradation product in peroxide condition

Materials and methods:
CRB was a gift sample obtained from a reputed manufacturing unit in Hyderabad. HPLC-grade Acetonitrile, Trifluoroacetic acid, sodium hydroxide, hydrochloric acid and hydrogen peroxide were procured from Merck, India. All other chemicals were of analytical-grade. HPLC grade water was Obtained from milli-Q water purification system (Millipore, Milford, USA).

Instrumentation and chromatographic conditions:
Waters Acquity UPLC instrument equipped with a binary solvent manager pump where Pump-A is used for aqueous solvent manager and Pump-B is used as organic solvent manager with four line options, an auto sampler, a column manager with 3 column switch over valve and PDA detector operated with Empower-3 software. The elution and separation was performed using Acquity; UPLC, CSH; C-18; 100 x 2.1mm; 1.7 µm column. with the mobile phase consisting of 0.1% Trifluoro acetic acid in water and 0.1% Trifluoro acetic acid in acetonitrile in a gradient elution mode within a short run time of 6.0 minutes at a flow rate of 0.4 ml/min with column temperature at 25°C. injection volume of 1.0µL, and wave length of 250 nm. Mobile phase(50:50 v/v) was employed as diluent. The system allowed equilibrating using mobile phase for 10 min prior to first injection. The method development, validation and stress degradation samples were analyzed and results were reported using empower-3 software.
Mobile phase, Stock and working standard solution preparation methods:
Preparation of buffer-A: Dissolved accurately 1.0 ml of Trifluoro acetic acid in 1000 ml of milli-q water and mixed well. This solution was sonicated and degassed to remove dissolved particles.
Preparation of buffer-B: Dissolved accurately 1.0 ml of Trifluoro acetic acid in 1000 ml of Acetonitrile, sonicated and mixed well.

Preparation of standard solution: A working standard stock solution of Crisaborole was prepared by dissolving standard equivalent to 100 mg of Crisaborole into 100 ml volumetric flask, to this added 60 ml of diluent and sonicated for 5 minutes and then take 10ml of above solution into 50ml volumetric flask containing 20ml of diluent further diluted to the volume with diluent to have a solution concentration of 200 ppm.
Preparation of diluted standard:
Diluted 1ml of the standard stock solution to 100 ml with diluent and mixed well, further diluted 5 ml of the resulting solution to 50 ml with diluent. The Obtained solution is of 1.0 ppm.
Preparation of sample solution: Transfer 100 mg of Crisaborole sample into 100 ml volumetric flask and added 60 mL of diluent and sonicated in ultrasonic bath for 20 minutes with intermediate shaking and diluted to the volume with diluent. Take 10ml of above solution into 50ml volumetric flask containing 20ml of diluent further diluted to the volume with diluent to have a solution concentration of 200 ppm. Filter the solution through 0.45 µm nylon membrane filter by discarding 4 ml of filtrate and injected the same solution (0.2 mg/ml).

Specificity and forced degradation studies (FDS)
Specificity of the test method was evaluated by analyzing standard substance against potential interferences. The blank, degradant samples and CRB standard were injected individually. The specificity of method was evaluated in presence of compound and degradants.
The stress conditions studied include acidic hydrolysis (2N HCl), basic hydrolysis (0.5N NaOH), peroxide oxidation (1% H2O2),thermal (120˚C) and photo light(254nm). For heat and light studies, the study period was 2 days where as acid, base and peroxide the test period was 6h. Peak purity of degradation samples of CRB was inspected using a photo diode array detector. CRB assays were performed by comparison with standard and mass balance (%assay +%impurities + %DPs) were calculated for stress samples. The degradation samples were injected into an UPLC instrument equipped with PDA detector to check the peak purity and homogeneity across the peak.

Method validation
The developed method was validated by determining and quantifying the CRB in accordance with ICH guidelines.
System suitability

The system suitability test was performed throughout the validation studies by injecting 0.2 mg. mL-1 of CRB standard solution. The USP Tailing, USP plate count and peak purity of CRB was checked on each parameter and each day of the validation.

Precision

The repeatability of the method analysed by injecting five individual test preparations of CRB standard (0.2 mg. mL–1). The intermediate precision was evaluated with same concentration solutions as used for method precision, but prepared separately on a different day by different analysts. This was checked for two days with two different analysts. Precision at LOQ levels was determined by injecting five preparations of CRB standard solution at a concentration of LOQ level. The %RSD of areas of CRB was calculated for the precision studies.

Limit of quantification and detection (LOQ & LOD)

The LOQ is defined by the signal to noise ratio (S/N) that should be ≥ 10 for CRB. The LOD was identified by evaluating S/N ratio for diluted solutions with the criteria of S/N ≥ 3.0 for CRB these values were evaluated by analyzing a series of diluted samples with known concentrations.

Linearity

Linearity of the proposed chiral method was established by analyzing series of 6 calibration samples of CRB ranging from LOQ - 150% at specification level (0.2 mg/mL) at different concentration ranges. CRD standard were prepared from 100 - 3000 µg. mL–1. The regression curve plotted with peak area vs concentration, using least square technique. The correlation coefficient (r2), slope and Y-intercept of CRB was analyzed from calibration plot.

Accuracy

This was determined by spiking known concentration of CRB sample. The accuracy was evaluated as % recovery. The study conducted in triplicates at 50, 100 and 150% and % recovery of CRB evaluated.

Robustness

The robustness study was carried out to check the influence of small variations on the system suitability criteria in the optimized chromatographic conditions. The factors chosen for this study are flow rate (± 0.1 ml/min), Mobile phase composition of organic phase concentration (± 10.0%), and temperature variation (± 5°C). In all the above varied conditions, the other parameters were kept constant. Standard solution of CRB (0.2 mg.mL-1) was injected for five times in all the above modified conditions and checked for resolution, retention and %RSD for CRB.

Solution stability

Solution stability of CRB was established by storing the solution (in capped container at room temperature and a standard solution of CRB (0.2mg.mL-1) for 72 h. The impurity content and system suitability were checked.

Results:

Method development and optimization

In order to estimate the CRB in bulk drugs with stability indicating assay method, significant number of methods screenings was performed based on compound structure. During initial developments different percentages of organic modifiers as mobile phase, various column stationary phases (CSPs) containing different C18 and end capping and different flow rate were examined. Initial screening with mentioned parameters was carried out to determine best suitable column and mobile phase that provides accurate method for routine analysis in laboratory.

Initially, the peak shape was broad and long run time using isocratic mode. In order to shorten the run time, gradient separation mode was optimized with good peak shape and elution. Optimal separation was attained on Acquity UPLC Xselect CSH C18 column with dimensions 100*2.1 mm, 1.7 µm. With column temp 25°C in gradient elution was executed using the combination of 0.1% Trifluoro acetic acid in milli-q water buffer (pH~ 1.9) and 0.1% Trifluoro acetic acid in acetonitrile as organic modifier at a flow rate of 0.4 mL/min. The short run time about 6.0 mins with UPLC detection was carried in PDA. Gradient program was cited in Table-1.

Time (minutes)	Flow rate (ml/min)	% of mobile phase-A	% of mobile phase-B
0.0	0.4	70	30
4.0	0.4	10	90
6.0	0.4	10	90
6.1	0.4	70	30

Tab-1: Gradient Program for UPLC method

Method Validation

Specificity and FDS:

CRB standard were injected to confirm the retention time, USP tailing and plate count of column. The specificity results captured in Table 2.

During forced degradation studies CRB sample was exposed to 2.0N HCl, 1% H2O2, 0.5N NaOH at room temperature with continuous constant stirring for 4-10 h individually. The CRB showed no degradation under Acidic, thermal and UV conditions, while significant degradation observed with 1% H2O2 and 0.5 N NaOH. Representative chromatograms for the degradation samples were shown in Fig. 3. The major degradation product was formed about 100% conversion in 1%H2O2 condition at 1.92 min. In basic conditions around 38 % of degradant observed. All these degradation impurities were well separated from CRB peak and the peak purity has been evaluated to check for any interference. CRB peak in stressed samples well separated with degradant products. All peaks were homogeneous and spectrally pure. It also showed that no degradants was co-eluted with the CRB standard peak was not observed in any stressed samples. The developed method was specific in presence of degradants with CRB peak. The

mass balance of all stress samples was close to 100.0% which indicate that there are no other by-products formed during degradation apart from DP impurities. (Table 2)

S.No	Stress condition	% Assay Drug remained	% impurities
1	5 mL 2N HCl/10h,RT°C	99.90	No degradation
2	5 mL 0.5N NaOH/6h,RT°C	62.17	37.5%
3	5 mL 1% H2O2/1h, RT°C	Not detected	100%
4	120 °C_48 Hrs	100.53	No degradation
5	Photolytic stability(UV)	100.30	No degradation

Tab-2: Specificity and Degradation studies for Crisaborole.

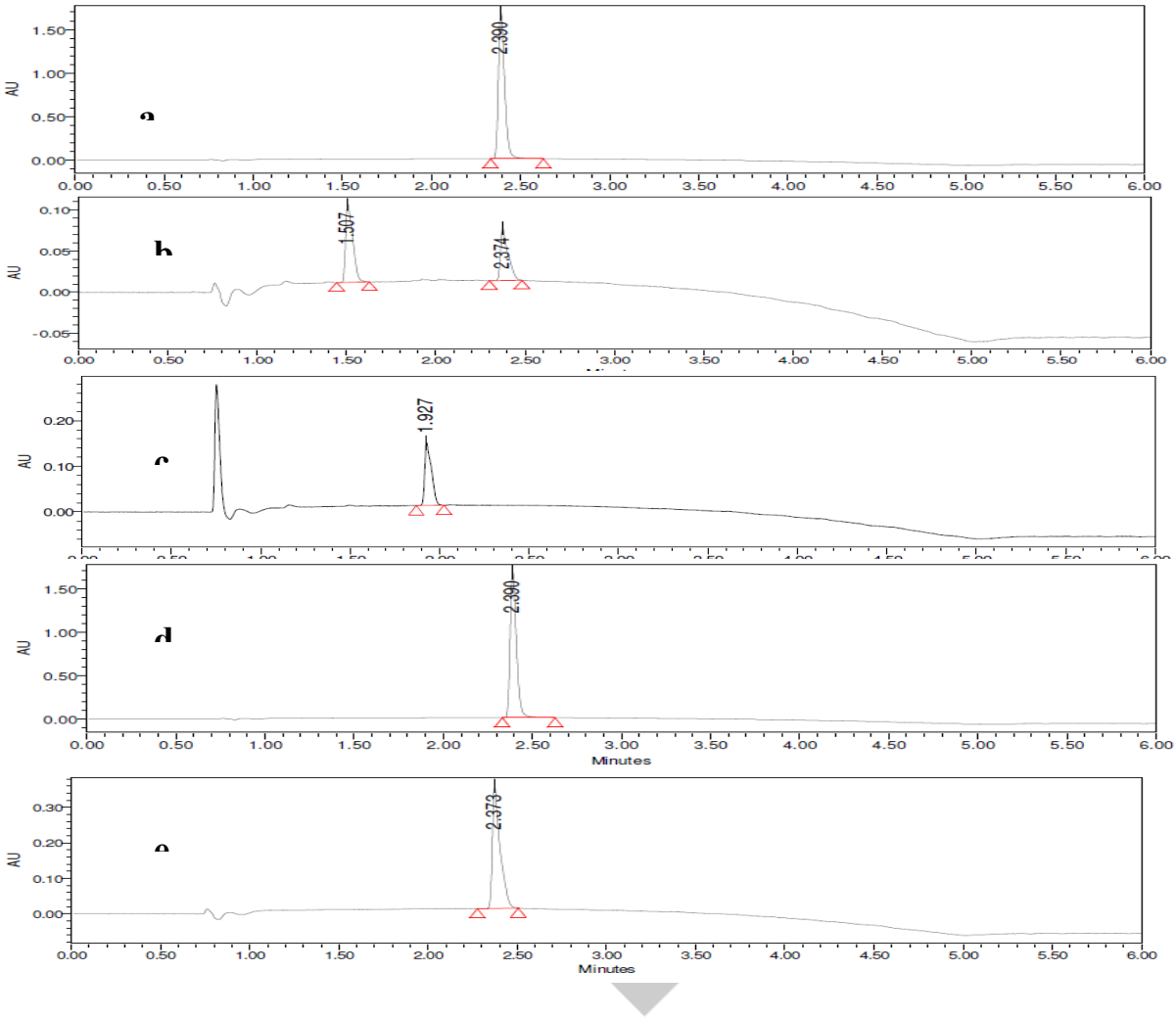

Fig.2. Typical degradation chromatograms of (a) Acidic (b) Base (c) Oxidative, (d) UV and (e) Thermal conditions.

System suitability:

System suitability solution was prepared by assay concentration of standard at the specification level (0.2 mg/ml) and injected to evaluate the system suitability of the method and found that Crisaborole retention time of 2.39 minutes. Chromatogram of CRB was illustrated in fig-2. The system suitability results were given in table-2. The developed UPLC method was found to be specific for Crisaborole in the proposed method. Standard solutions of Crisaborole working standard was prepared as per procedure and were injected five times into the UPLC system. The system suitability parameters were evaluated from standard chromatogram. The % RSD for area count and retention time, tailing factor and theoretical plates from five replicate injections are within range and results were shown in Table 3 and Figure 3.

Compound Name	RT	RRT	USP Tailing	USP Plate count	Purity1Angle	Purity1Threshold	Purity1 Flag
CRB	2.39	1.00	1.08	165497.54	0.144	0.998	No

CRB	System suitability
No of Injs	**Area**
Inj-1	8574961
Inj-2	8556620
Inj-3	8421041
Inj-4	8649510
Inj-5	8632141
Average	8566854.60
SD	90175.24
% RSD	**1.05**

Tab-3: System suitability results (RT-Retention time, RRT-relative retention time)

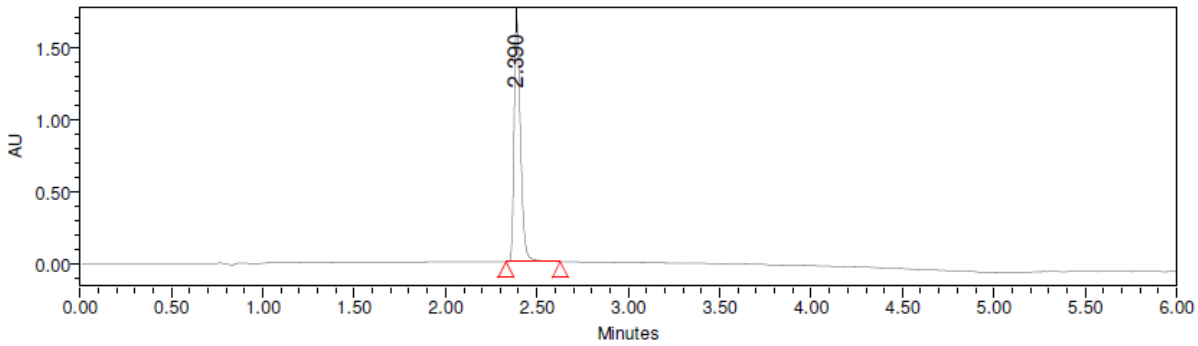

Fig.3. System suitability chromatogram.

Precision:

The precision of a method determines the closeness of agreement between a series of measurements of the same sample. The intraday and interday precisions were carried out 5 times at concentration of 0.2 mg/mL and the %RSD were found to be 1.11 to 0.73%, respectively. The precision results were within the accepted limits of ≤ 2.0 % RSD which proves that the method was precise. The results were tabulated in Table-4. Hence the developed method is precise for its intended use.

CRB	System precision	Method precision	Ruggudness-1	Ruggudness-2
No of Injs	**Area**	**Area**	**Area**	**Area**
Inj-1	8802631	8564213	8592411	8695312
Inj-2	8795621	8509736	8600240	8655120
Inj-3	8802244	8672194	8559924	8794528
Inj-4	8597301	8609870	8587821	8642390
Inj-5	8657432	8632461	8422940	8599864
Average	8731045.80	8597694.80	8552667.20	8677442.80
SD	97044.12	62779.05	74095.10	73779.62
% RSD	**1.11**	**0.73**	**0.87**	**0.85**

Tab-4: Method precision and intermediate precision data.

LOD and LOQ:

LOD is a limit test parameter and it is a test to determine whether the analyte concentration was present within the specification limit or not. LOQ is a parameter for quantitative assay used particularly for determination of impurities or degradation products as it used for minimum concentrations of analyte in sample. The LOD and LOQ were found to be 0.006 and 0.01 respectively and the % RSD for LOQ precision of CRB was 1.8%, which proves the method was sensitive. The LOQ precision values were mentioned in Table.5.

CRB	
No of Injs	**Area**
Inj-1	55436
Inj-2	54297
Inj-3	56047
Inj-4	56112
Inj-5	53972
Average	55172.80
SD	990.52
% RSD	**1.80**

Tab-5: LOQ precision establishment for Crisaborole.

Linearity and range:
Linearity of the developed method was evaluated for six different levels of Crisaborole assay. The concentrations ranged from LOQ to 150 % of assay specification limit. The respective peak area was recorded and plotted against standard concentration and the graph resulted in straight line. The correlation coefficient, slope, intercept and % Y-intercept values were calculated and tabulated for Crisaborole. The compiled results were tabulated below in table-6. Correlation Co-efficient was found to be 0.9995 and the linearity graph shown in Fig.5.

S.No	Concentration (%)	Area response of CRB
1	0.01	59664
2	0.50	4575411
3	0.75	6641944
4	1.00	8666854
5	1.25	11033574
6	1.50	13101781
Correlation coefficient		**0.9995**

Tab-6: Linearity results for Crisaborole.

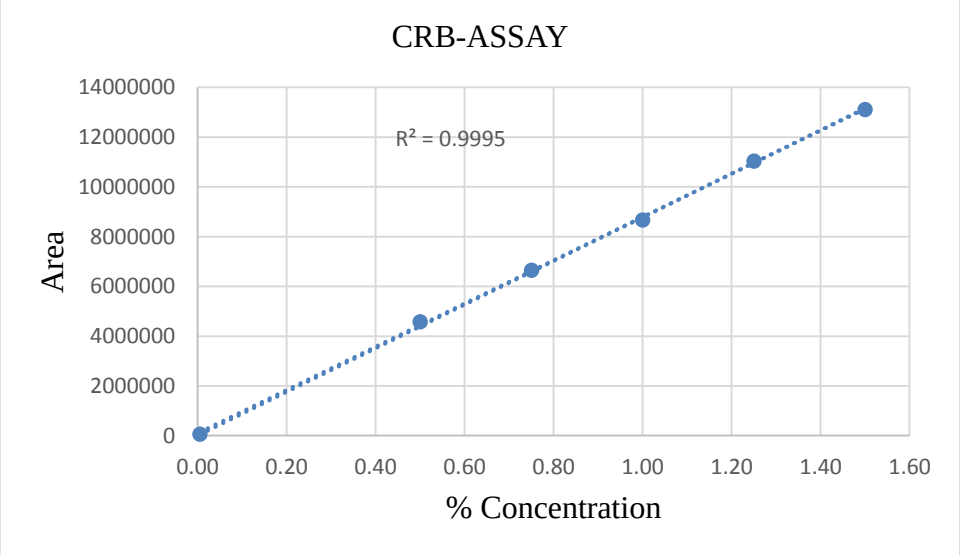

Fig.3. Linearity graph for CRB assay.

Accuracy:
Recovery studies were performed to judge the accuracy of the test method. The study was evaluated by spiking the known quantity of CRB at various levels on the blank. From the amount of CRB found the % recovery was calculated. Recovery was performed at four different levels ranging from LOQ to 150 % of the specification level. The % recovery of CRB was found to be within the

acceptance criteria of 98.0% to 102.0%. So the method is accurate for the determination of Crisaborole quantification. Good recovery results obtained for the developed method indicates that this method can be used for regular quality control assay test for Crisaborole. The mean recovery values for the CRB assay were tabulated in table-7.

S.No	Conc level	% Mean recovery ± SD of CRB
1	LOQ	101.16
2	50%	100.58
3	100%	100.40
4	150%	100.05

Tab-7: Accuracy results for Crisaborole

Solution and mobile phase stabilities

No significant variations were observed for CRB standard solution (0.2mg/mL) during stability study, % RSD was < 2.0 for CRB. No unknown peaks were observed in storage conditions hence found stable upto 72 h (Table-8).

CRB	0hrs	36hrs	72hrs
No of Injs	Area	Area	Area
Inj-1	8574961	8674155	8497602
Inj-2	8556620	8602971	8505531
Inj-3	8421041	8695438	8597637
Inj-4	8649510	8532970	8639885
Inj-5	8632141	8639745	8600971
Average	8566854.60	8629055.80	8568325.20
SD	90175.24	64163.25	63227.01
% RSD	**1.05**	**0.74**	**0.74**

Tab-8: Solution stability results for Crisaborole

Robustness:

The robustness of this procedure was assessed by evaluating its ability to remain unaffected by deliberate variations such as organic modifier, flow rate and temperature. In all the modified conditions the system suitability results (%RSD, Resolution and Retention) were in acceptable range indicating the reliability of the proposed method. (Table-9).

CRB	Initial	Flow Decrease	Flow Increase	Organic Decrease	Organic Increase
No of Injs	Area	Area	Area	Area	Area
Inj-1	8574961	8894521	8679541	9027411	8502746
Inj-2	8556620	8866979	8577330	9109745	8593267
Inj-3	8421041	8739210	8609546	8993567	8694721
Inj-4	8649510	8912870	8765329	8902874	8559907
Inj-5	8632141	8966215	8539852	8899672	8652974
Average	8566854.60	8875959.00	8634319.60	8986653.80	8600723.00
SD	90175.24	84607.99	89434.49	88664.08	75641.95
% RSD	**1.05**	**0.95**	**1.04**	**0.99**	**0.88**

Discussion and conclusion:

The present work established stability indicating assay UPLC method for the first time to determine the CRB with good separation in degradation conditions and quantification of CRBin bulk and formulation drugs. The developed assay method showed the separation of unwanted degradation impurities from the peaks of interest (CRB) ensuring the selectivity and accuracy of the detection. This method was able to detect all the degradation impurities. The proposed method was found with good resolution and short run time (6.0minutes). This method can be applicable and used in process development, stability analysis and quality control analysis of CRB bulk drug and formulations for detecting, quantification and other possible degradation products. This quantification method by UPLC is the time and cost effective and also environmentally eco-friendly than traditional regular HPLC methods with longer run time. This method is a mass compatible method easy to transfer in LCMS.

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