A NOVEL RIVAROXABAN DEGRADATION IMPURITY DETECTION BY RP-HPLC, EXTRACTION BY PREPARATORY CHROMATOGRAPHY, AND CHARACTERIZATION BY LC-MS, NMR, AND FT-IR: ANALYSIS OF NOVEL IMPURITY IN BATCH SAMPLES AND TABLETS OF RIVAROXABAN

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ABSTRACT
A novel rivaroxaban degradant was obtained at 0.45% w/w level during the “RP-HPLC” analysis in the acetic acid-exposed rivaroxaban sample. This novel rivaroxaban degradant has a relative retention period (RRT) of approximately 0.95 min. This 0.95 RRT impurity was recognized by LC-MS and then characterized by \textsuperscript{1}H NMR, \textsuperscript{13}C NMR, NMR HSQC, NMR HMBC and NMR D\textsubscript{2}O exchange analyses, and FT-IR techniques. Employed Zorbax XDB C18 eclipse as the main column and Ghost-Guard-LC, ANP00001 as the Ghostbuster column for 0.95 RRT molecule analysis. The mobile phase, phosphate buffer, and acetonitrile mixture, with a simple gradient, were provided to a column at a flow of 1.0 ml/min rate. Samples were evaluated employing a wavelength of 250 nm and injected sample at a volume of 3.0 \textmu L. Good sensitivity was obtained (LOD –0.02% concentration and LOQ – 0.05% concentration) with levels extremely less than allowable limits (0.15%). In the concentration ranges of 0.045% (LOQ) – 0.225% (150% of permissible limits), the technique demonstrated the best linearity for 0.95 RRT molecule analysis, with coefficients of determination of 0.9958. The recovery rate ranged from 93.2% to 105.7%. This approach is shown to be capable of determining 0.95 RRT impurity levels in three rivaroxaban batch samples and one rivaroxaban commercial tablet.

Keywords: Rivaroxaban, 0.95 RRT Impurity, Stress Exposure, NMR Data, FT-IR Data, LC-MS Data.

INTRODUCTION
Rivaroxaban (RVB) is a significant breakthrough in the area of oral anticoagulant medicine.\textsuperscript{1} Patient, healthcare infrastructures, and the community are all burdened by thrombosis-related disorders. Vein deep thrombosis, myocardial infarction, pulmonary embolism, stroke, as well as death are all possible outcomes of thrombosis. RVB works mainly by blocking coagulation Factor Xa in the bloodstream to avert and cure thrombosis.\textsuperscript{2} RVB has been authorized across the world to avoid and manage thrombosis-related diseases after a comprehensive and unique clinical research program involving over 75,000 patients.\textsuperscript{3,5} In the literature, a range of different synthetic processes for the manufacturing of RVB have been documented in journal papers including patents.\textsuperscript{6,12} Ramisetti & Kuntamukkala described LC-PDA-MS/MS,\textsuperscript{13} Rajan and Basha stated RP-UPLC,\textsuperscript{14} Arous et al., reported HPLC/UV\textsuperscript{15}, and Yashpal Singh et al. described HPLC/UV\textsuperscript{16} proposed methodologies for assessing RVB process-correlated impurities in pure drug and formulations of RVB. A novel rivaroxaban degradant with a relative retention period of approximately 0.95 with regard to the RVB was obtained at 0.45% w/w level during the “RP-HPLC” analysis in the acetic acid exposed sample. However, a thorough search of the literature finds that no publications on the quantitative assessment and characterization of this impurity have been published yet. In this article, we investigated and presented the production of degradation RVB impurities after stability storage. RVB was submitted to stress conditions inside this investigation, as specified by the “ICH”.\textsuperscript{17} We
Rivaroxaban stability samples were investigated in this study, and one new impurity was detected besides the recognized impurities at a relative retention period of approximately 0.95 with regard to the RVB. As this is a novel new impurity, hence for the characterization of this impurity, LC-MS, Fourier transform infrared spectroscopy and nuclear magnetic resonance techniques are required. The current study shows how we identified new impurity through RP-HPLC, isolated the new impurity through preparative liquid chromatography, and deduced the structure of the 0.95 relative retention period impurity with LC-MS, Fourier transform infrared spectroscopy and nuclear magnetic resonance techniques.

**EXPERIMENTAL**

**Solvents and Chemicals**
Emparta grade KH$_2$PO$_4$, potassium bromide, orthophosphoric acid, sodium hexane sulfonate, ammonium formate, ammonium carbonate, acetic acid, HPLC grade methanol, and acetonitrile were procured from “Merck limited”, Germany. The DMSO-$d_6$ solution was obtained from “Cambridge Isotope Laboratories”, US. The Millipore purification technology was utilized to generate pure Milli-Q water.

**High-Performance Liquid Chromatography-Identification of New Impurity**

**Solution A**
Measured KH$_2$PO$_4$ (1.36 gm) into a beaker holding Milli Q water (500 ml), added orthophosphoric acid (200 ml), diluted to 1000 ml using Milli Q water, then filtered as well as degassed through a 0.45 µ porous filter membrane.

**Mobile phase A**
Further dissolved sodium hexane sulfonate (1.0 gm) in above solution A. Then again filtered as well as degassed through a 0.22 µ porous filter membrane. Mixed methanol (50 ml) with mobile phase A (950 ml).

**Mobile Phase B**
This includes pure acetonitrile.

**Diluent**
Acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes.

**Sample for Analysis**
In a volumetric flask (50 ml), RVB (25.32 mg) was mixed in acetic acid (5 ml) solution and maintained at 80 °C for 12 hr with constant stirring, then neutralized using 10% NaOH solution to attain a pH unit value of 7.0.

**Analyzing Conditions**
For this investigation, a “Waters” HPLC system was deployed. Employed Zorbax XDB C18 eclipse (3.0 mm × 150 mm with 3.5 µm particle dimensions) as the main column and Ghost-Guard-LC, ANP00001 (4.6 mm × 30 mm dimensions) as the ghostbuster column. Mobile phases A and B with simple gradient program (0 min: A 98 vol%: B 2 vol%, 2 min: A 98 vol%: B 2 vol%, 8 min: A 84 vol%: B 16 vol%, 25 min: A 64 vol%: B 36 vol%, 37 min: A 20 vol%: B 80 vol%, 45 min: A 20 vol%: B 80 vol%, 48 min: A 98 vol%: B 2 vol%, 60 min: A 98 vol%: B 2 vol%) was provided to column at a flow of 1.0 ml/min rate. The temperatures at columns were preset at 60 °C. Samples were evaluated employing a wavelength of 250 nm and injected sample at a volume of 3.0 µL. Run time included 60 mins.

**Stress Stability Tests**
Stability testing is required by the ICH standards in order to identify possible degradation products or explain the RVB substance's stability properties (ICH guidelines, 2003). Acid, thermal, base and oxidative exposure studies were conducted on RVB.

**Real-Time Stability Test**
This was done on two batches (RB/A0227/STG-05/19/189 and RB/A0231/STG-05/02/029) of RVB samples. In a 100 ml size volumetric flask, weighed RVB (50.17 mg) and made up to the mark (100 ml) with diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes) and dissolved, and mixed up well. These RVB samples were stored at 25°C and 60% relative humidity for
intermediate stability testing, and at 40 °C and 75% relative humidity for accelerated stability testing. At the 1\textsuperscript{st}, 2\textsuperscript{nd}, 3\textsuperscript{rd}, and 6\textsuperscript{th} months, the stored samples were evaluated using analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity.”

**Acid Degradation**

In a 100 ml size, a volumetric flask weighed RVB (50.27 mg), added 1N HCl (10 ml), and stirred for 24 hr at 50°C. Next to 24 hr, added 1N NaOH (10 ml), made up to the mark (100 ml) with diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes) and dissolved, and mixed up well. This sample was analyzed by deploying analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity”.

**Base Degradation**

In a volumetric flask (100 ml), RVB (50.17 mg) was mixed in 1.0N NaOH (10 ml) solution and maintained at 50 °C for 24 hr with constant stirring, then neutralized using 1.0N HCl solution to attain pH unit value 7.0. This degraded sample was made up to the mark (100 ml) with diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes), dissolved, and mixed up well. This sample was analyzed by deploying analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity”.

**Thermal Degradation**

Weighed RVB (100 mg) into a Petridish and kept it back at 105 °C in the oven. Following 24 hr, weighed RVB (50.20 mg) was into a volumetric flask (100 ml), made up to the mark of 100 ml with diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes), mixed well, and analyzed with conditions in sector “High-performance liquid chromatography-identification of new impurity”.

**Peroxide Degradation**

In a 100 ml size, a volumetric flask weighed RVB (50.28 mg), added 3% concentrated peroxide (10 ml), and stirred for 24 hr at 50°C. Next to 24 hr, made up to the mark (100 ml) with diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes), dissolved, and mixed up well. This sample was analyzed by deploying analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity”.

**Isolation of New Impurity – Preparative Liquid Chromatography**

**Mobile Phase A**

Measured ammonium bicarbonate (0.78 gm) into a beaker holding Milli Q water (500 ml), diluted to 1000 ml using Milli Q water, then filtered as well as degassed through a 0.45 µ porous filter membrane.

**Mobile Phase B**

This includes pure acetonitrile.

**Sample Solution**

In a 10 ml size volumetric flask, correctly measured 1000 mg of acetic acid degraded RVB sample, added dimethyl sulfoxide (5.0 ml) to dissipate the RVB, and bring up to the marking (10 ml) with acetonitrile solvent.

**Methodology**

For the isolation of impurity, a “Waters” HPLC system was deployed. Employed Xbridge C18 Prep (19 mm × 250 mm with 5.0 µm particle dimensions) as the main column. Mobile phases A and B with a simple gradient program (0 min: A 95 vol%: B 5 vol%, 5 min: A 10 vol%: B 90 vol%, 12 min: A 5 vol%: B 95 vol%) were provided to the column at a flow of 20 ml/min rate. The temperatures at columns were
preset at 60 °C. Samples were evaluated employing a wavelength of 250 nm and injected sample at a volume of 100 µl. Run time included 12 mins.

**LC-MS-Analysis of New Impurity**

**Mobile Phase A**
Measured ammonium formate (0.315 gm) into a beaker holding Milli Q water (500 ml), diluted to 1000 ml using Milli Q water, then filtered as well as degassed through a 0.45 µ porous filter membrane. Further dissolved formic acid (1.0 ml) in the above solution (10 mM ammonium formate). Then again filtered as well as degassed through a 0.22 µ porous filter membrane. Mixed methanol (50 ml) with mobile phase A (950 ml).

**Mobile Phase B**
This includes pure acetonitrile.

**Diluent**
0.1% formic acid made in acetonitrile solvent and water are blended in 40:60 v/v proportion volumes.

**Sample**
Fraction recovered from preparative chromatography was utilized for LC-MS analysis.

**Analyzing Conditions**
Waters Premier Quattro Triple Quadrupole LC-MS instrument loaded with MasslynxSoftware was exploited to analyze the fraction recovered from preparative chromatography. Mass settings employed included: positive mode ESI, source temperature of 120°C, desolvation temperature of 350°C, capillary volts of 3.2 KV, cone volts of 20.0V, RF lens volts of 0.2V, cone gas flow stream at 140 L/hr, and desolvation gas flow stream at 1000 L/hr. Employed Zorbax XDB C18 eclipse (3.0 mm × 150 mm with 3.5 µm particle dimensions) as the main column. Mobile phases A and B with simple gradient program (0 min: A 98 vol%: B 2 vol%, 2 min: A 98 vol%: B 2 vol%, 8 min: A 84 vol%: B 16 vol%, 25 min: A 64 vol%: B 36 vol%, 37 min: A 20 vol%: B 80 vol%, 45 min: A 20 vol%: B 80 vol%, 48 min: A 98 vol%: B 2 vol%, 60 min: A 98 vol%: B 2 vol%) was provided to column at a flow of 1.0 ml/min rate. The temperature at column preset at 60 °C was used. Injected sample at a volume of 3.0 µl. Run time included 60 min.

**Nuclear Magnetic Resonance**
The solvent, dimethyl sulfoxide (DMSO-d6), was used for the NMR analysis experiments. “Bruker 400 MHz NMR spectrometer” was exploited for the NMR analysis of new impurity. The number of scans was16 for ¹H NMR analysis, 1500 for ¹³C NMR analysis, 16 & 254 for NMR HSQC analysis, 16 & 30 for NMR HMBC analysis, and 16 for NMR D₂O exchange analysis. The pulse programs were zg30, zgpg30, and zg30 for ¹H NMR, ¹³C NMR, NMR HSQC, NMR HMBC, and NMR D₂O exchange analyses, respectively.

**Fourier Transform Infrared Spectroscopy**
“Perkin Elmer” made “spectrum two FT-IR Spectrometer” was deployed in new impurity FTIR analysis. PCI Analytics hydraulic automatic press Cap-15 Ton was deployed for pellet preparation. RVB (2 mg) was dispersed in finely powdered and dehydrated potassium bromide (400 mg). Ground this combination, distributed it evenly on a die, and pressed it into a small disc at a pressure of up to 800 MPa. Using comparable weights and settings, developed the RVB working/CRS standard disc. Documented the FITR spectra of RVB between 450 cm⁻¹ and 4000 cm⁻¹.

**General Approach for Analyzing 0.95 RRT Impurity**
Different dilutions of 0.95 RRT impurity (0.045%, 0.075%, 0.150%, 0.1875% and 0.225% concentrations) were made in diluent (acetonitrile solvent and solution -A are blended in 40:60 v/v proportion volumes), injected onto the HPLC system and analyzed analyzed with conditions in sector “High performance liquid chromatography-identification of new impurity”. Injections of every concentration (0.045%, 0.075%, 0.150%, 0.1875% and 0.225%) were charted on 0.95 RRT impurity calibration curve (peak area vs concentration). The 0.95 RRT impurity calibration curve’s intercept,
slope, and also correlation coefficient were calculated. The 0.95 RRT impurity content could be estimated with the use of its regression equation or its calibration curve.

**RVB Batch and RVB Tablet Analysis for 0.95 RRT Impurity**  
Three RVB sample batches (RB/A0243/STG-05/04/013; RB/A0231/STG-05/02/029 and RB/A0227/STG-05/19/189) and one RVB commercial tablet with 2.5 mg RVB strength (Batch Number: SA03992, Cipla Ltd., India) were analyzed for 0.95 RRT impurity content deploying analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity”. The 0.95 RRT impurity content in RVB sample batches and RVB commercial tablets can be estimated with the use of its regression equation or its calibration curve.

**RESULTS AND DISCUSSION**

**Stress Stability Tests**

Acid, thermal, base, and oxidative exposure studies were conducted on RVB. Owing to the outcomes from the degradation exposure experiments, RVB deterioration was seen more in the base condition, while RVB was more persistent in acid, peroxide, and thermal exposure scenarios. The chromatograms associated with stress exposure tests are also provided (Fig.-1).

**Fig.-1:** Impurity Profile Chromatograms in Stress Exposure Tests
Detection of New Impurity

The sample RVB solution specified beneath subsection “Sample for analysis” of section “High-performance liquid chromatography-identification of new impurity” was injected and analyzed. An undisclosed impurity was detected together with identified impurities with a relative retention period of approximately 0.95 min relative to the RVB peak on the chromatogram (Fig.-2), and a similar impurity was also seen in expedited stress conditions such as base as well as peroxide conditions. This 0.95 RRT impurity was not observed in real-time stability sample testing. At 0.45 percent, this 0.95 RRT impurity is generated, which is more than the single highest unknown impurity threshold. Consequently, it was extracted using preparatory HPLC and subsequently characterized using the LC-MS, NMR, and FT-IR.

Fig.-2: Impurity 0.95 RRT Chromatogram in Acetic Acid Exposure Test

0.95 RRT Impurity Isolation

The acetic acid degraded RVB solution was loaded into the preparatory LC column and isolated. Impurity fractions with a window period of 15-16 min were collected and then concentrated on Rota vapor to evaporate the organic component solvent. The concentrate fraction was placed into a preparatory LC column, and the eluent was processed with water to eliminate the isolation buffer. Lastly, the column was cleansed in a 20:80 v/v mixture of water with acetonitrile. The unidentified 0.95 RRT impurity collected via the preparatory LC isolation method was injected into the HPLC device system and analyzed to authorize the retention time of 0.95 RRT impurity (Fig.-3).

Fig.-3: Confirmation of Retention Time of 0.95 RRT Impurity

Structural Elucidation of 0.95 RRT Impurity

$^1$H and $^{13}$C NMR spectra of impurity were recorded in DMSO-$d_6$. The $^1$H and $^{13}$C NMR chemical shifts of the DMSO-$d_6$ were 2.50 and 39.5 ppm referenced to TMS respectively. The procedure to assign the NMR data of impurity at 0.95 RRT; N-((3-(4-acetamidophenyl)-2-oxooxazolidin-5-yl)methyl)-5-chlorothiophene-2-carboxamide (Fig.-4) is explained in detail here.

Fig.-4: Structure of 0.95 RRT Impurity
We herein report our efforts on the structural confirmation of Impurity using 1D and 2D NMR spectroscopy. To assign chemical shift values and multiplicities to each proton and carbon atom labeling was assigned to impurity. The chemical shift value of each proton assigned to Impurity carbon at the 15th position having 2 protons was most informative because significant differences were observed in this region. Chemical shift at 14th position having 2 protons resonated at same chemical shift and it was further confirmed from carbon at 13th position having one proton. In 1D 1H NMR spectrum of Impurity contained 4 aromatic methine protons were attributed to the aromatic ring, because of their complex splitting pattern (two multiplets). These protons were identified as 2-H & 6-H and 3-H & 5-H respectively. Two protons (14-2H) resonated at the same chemical shift 3.61 (one multiplet). It was very interesting because one proton (15-Ha) resonated at 3.82 and the second proton(15-Hb) resonated at 4.11 as two multiplets. The single proton at 13-H resonated at 4.84 and showed a complex splitting pattern due to adjacent 14-2H, 15-Ha & 15-Hb. Two labile 7-H & 16-H protons at chemical shift values 8.99 & 9.95 were confirmed by D₂O experimentation. The chemical shift value of the carbon atom at 71.6 ppm was subsequently determined from HSQC & HMBC spectra. The ESI-MS mass spectra of the 0.95 RRT impurity displayed the peak at m/z 394.40 paralleling to (M+H)+, confirming the molecular formula, C₁₇H₁₆ClN₃O₄S. Furthermore, FT-IR spectrum measurements show the confirmation of this 0.95 RRT impurity. Below are the FTIR data (Table-5) and FTIR spectra of 0.95 RRT impurity. The 0.95 RRT impurity is chemically called N-((3-(4-acetamidophenyl)-2-oxooxazolidin-5-yl)methyl)-5-chlorothiophene-2-carboxamide based on these spectrum data.

**Table-5: IR Assignments for 0.95 RRT Impurity**

| S. No. | Wave number (cm⁻¹) | Assignment | Mode of vibration |
|-------|-------------------|------------|------------------|
| 1     | 1663              | C=O (Amide)| Stretching       |
| 2     | 1736              | C=O (Ester)| Stretching       |
| 3     | 3263              | -N-H       | Stretching       |

**Method Validation**

The analyzing conditions in the sector “High-performance liquid chromatography-identification of new impurity” was validated for the quantification of 0.95 RRT impurity in accord with the existing ICH guiding principles (ICH guidelines, 2005) for linearity, the limit of detection, precision, the limit of quantification, accuracy, selectivity, and robustness.¹⁸

**Sensitivity**

By injecting a range of 0.95 RRT solutions diluted with specified concentrations, the measures of LOQ and LOD for 0.95 RRT impurity at signal: noise value ratio of 10:1 and 3:1 were examined, respectively. The measures were 0.05% (LOQ) and 0.02% (LOD), indicating better sensitivity to quantify 0.95 RRT impurity.

**Linearity**

From a series of 0.95 RRT impurity concentrations varying from concentrations 0.045% (LOQ) to 0.225% (150% specification content), the linearity of 0.95 RRT impurity was evaluated content). The linear regression (least squares) was applied to evaluate the peak area of 0.95 RRT impurity vs its concentration, and the correlation coefficient measure of regression (r²) was > 0.9958, showing superior linearity in quantifying 0.95 RRT impurity. The linearity equation for 0.95 RRT impurity obtained was: Peak area of 0.95 RRT impurity = 49997 × concentration of 0.95 RRT impurity + 192.99.

**Specificity**

By injecting the mobile phase, standard 0.95 RRT impurity (0.15% concentration) solution, RVB sample (0.5 mg/ml) solution, and 0.95 RRT impurity (0.15% concentration) spiked RVB sample into the HPLC system, the HPLC process specificity for the assessment of 0.95 RRT impurity was established. The non-appearance of excipient peaks exploited to formulate RVB, components peaks exploited to formulate the mobile phase and RVB peaks in the acquired chromatograms (Fig.-5) indicate better specificity to quantify 0.95 RRT impurity.
Precision
Six unique 0.95 RRT impurity (0.15% quantity) solutions were employed to do the precision. The RSD (0.38%) of 0.95 RRT impurity’s peak areas was a smaller measure than 0.5%, suggesting that the procedure has superior precision in quantifying 0.95 RRT impurity.

Accuracy
Standard addition plus recovery studies in triplicate at four concentration levels i.e., LOQ (0.0448% concentration), 50% (0.0747% concentration), 100% (0.1493% concentration), and 150% (0.2240% concentration) of the specification concentration, were performed to verify the accuracy. The mean recoveries of 0.95 RRT impurities were between 97.9% and 102.4% (at LOQ range), 98.9% and 105.7% (at 50% specification range), 93.2% and 100.8% (at 100% specification range) and 97.2% and 98.8% (at 150% specification range). The measured recoveries indicting superior accuracy in quantifying 0.95 RRT impurity.

Application: Content assay of 0.95 RRT impurity in RVB Batch and Tablet samples
Three RVB sample batches (RB/A0243/STG-05/04/013; RB/A0231/STG-05/02/029 and RB/A0227/STG-05/19/189) and one RVB commercial tablet with 2.5 mg RVB strength (Batch Number: SA03992, Cipla Ltd., India) were analyzed for the 0.95 RRT impurity content. The 0.95 RRT impurity was not identified in RVB batch samples, however, 0.01 % (below detectable limits) of 0.95 RRT impurity was reported in the tablet RVB sample.

CONCLUSION
Real-time, acid, heat, base, and oxidative stress exposure experimentations were performed on RVB substance samples. The degradation product that was generated was examined. There was one unidentified impurity observed with a relative retention period of 0.95 in heat stress exposure experimentation. After isolating this 0.95 RRT impurity, it was characterized using LC-MS, NMR spectroscopy, and FT-IR spectroscopy. Relying on the available spectrum data, the most likely structure for 0.95 RRT impurity was postulated. All technique validation factors, such as linearity, precision, specificity, sensitivity, and accuracy, for the HPLC technique for 0.95 RRT impurity analysis were met satisfactorily. Additionally, the approach was effectively applied to assess the 0.95 RRT impurity content in three different RVB batch samples and one RVB commercial tablet, indicating that the approach might very well be applied to analyze 0.95 RRT impurity.

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