

# 1-(4-Aminophenyl)-3-Morpholinopiperidin-2-One: A Process Related Impurity of Apixaban- An Anticoagulant Drug

Vipin Kumar<sup>1,3</sup>, Sachin Kumar Sharma<sup>1</sup>, Govind Singh<sup>1</sup>, Shyambaran Dhuriya<sup>1</sup>, Lingaiah Balthu<sup>1</sup>, Sushilkumar<sup>2</sup>, Rashmi Devi<sup>2</sup>, Krishna Kumar Yadav<sup>2</sup>, Priyanka Mathur<sup>3</sup>, Sumit S. Chourasiya<sup>1,\*</sup>

<sup>1</sup>Department of Process Research and Development, IOL Chemicals and Pharmaceuticals Ltd., Barnala, Punjab, INDIA.

<sup>2</sup>Department of Analytical Research and Development, IOL Chemicals and Pharmaceuticals Ltd., Barnala, Punjab, INDIA.

<sup>3</sup>Bhagwant Global University, Kotdwar, Uttarakhand, INDIA.

## ABSTRACT

**Introduction:** Apixaban is an anticoagulant drug act by direct inhibition of factor Xa. Apixaban is used for the treatment and prevention of blood clots and stroke in people with nonvalvular atrial fibrillation. In this report, we describe the identification of a process-related impurity observed during the preparation of apixaban, an anticoagulant drug. **Objectives:** The primary objective of this study was to isolate, purify, and characterize an unknown process impurity observed during the synthesis of Apixaban. **Materials and Methods:** All the reagents and solvents required for the synthesis were obtained from commercial vendors and had the desired purity of >98%. Isolation of unknown impurity was carried out using Flash Chromatography, Puriflash 5.250 using acetonitrile/water as eluent in gradient mode. The column used in Puriflash is YMC- Triart Prep-C18 250×20 mm, 10 μ. NMR spectra of the synthesized compounds were recorded on Bruker 400 MHz spectrometers with TMS as the internal standard. Chemical shift is represented with δ in parts per million. Mass spectra were recorded with a XEVO-TQSmicro#QEE0233 LC Mass Spectrometer. The IR spectra of synthesized compounds were recorded on Perkin Elmer Spectrum IR ES Version 10.6.0 by using the KBr pellet method. The purity of apixaban and its intermediates was determined by HPLC using an area normalization method. **Results:** As a part of the investigation, we isolated the impurity by column chromatography with >96% purity and analyzed the sample of impurity by LC-MS, IR, and NMR spectroscopy. The LC-MS analysis coupled with IR and NMR analysis suggested that the impurity is 1-(4-aminophenyl)-3-morpholinopiperidin-2-one, generated during preparation of Apixaban intermediate (APN-05). The impurity is a reduced form of APN-05, possibly formed due to reduction of the ring double bond of dihydropyridin-2(1H)-one moiety of APN-05. The genotoxic assessment of the impurity revealed its probability to exhibit genotoxic properties.

**Keywords:** Apixaban, Process-related impurity, Structural alert, Genotoxic impurity, Root cause investigation.

## Correspondence:

**Dr. Sumit Sunil Chourasiya**

Department of Process Research and Development, IOL Chemicals and Pharmaceuticals Ltd., Barnala, Punjab-148101, INDIA.  
Email: sumitchourasiya@iolcp.com

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## INTRODUCTION

Anticoagulants are commonly used for the prevention and treatment of thromboembolic disorders.<sup>1</sup> Warfarin, heparin, and their derivatives are traditional anticoagulants.<sup>2,3</sup> These anticoagulants are associated with several drawbacks, including drug-drug and drug-food interactions, parenteral administration, and routine monitoring, suggesting the urgent need for the development of safe and efficacious oral anticoagulants.<sup>4</sup>

The direct inhibition of factor Xa is a newly identified mechanism for controlling coagulation.<sup>5</sup> In this category, apixaban has evolved as a safe, efficacious, and convenient molecule for controlling coagulation.<sup>6</sup> Apixaban was approved for medical use in the European Union in May 2011.<sup>7</sup> A New Drug Application (NDA) for the approval of apixaban was submitted to the US Food and Drug Administration (FDA) by Bristol Myers Squibb (BMS) and Pfizer jointly after the conclusion of the ARISTOTLE clinical trial in 2011.<sup>8</sup> On 13 March 2014 it was approved for the additional indication of preventing deep vein thrombosis and pulmonary embolism in people who have recently undergone knee or hip replacement.<sup>9,10</sup> Apixaban is sold under the brand name Elikvis as an anticoagulant medication. Other examples of direct Factor (FXa) inhibitors include rivaroxaban,<sup>11</sup> edoxaban,<sup>12</sup> and betrixaban<sup>13</sup> (Figure 1), which have gained approval for several indications, most notably, for the prevention and treatment of



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Venous Thromboembolism (VTE) and for the prevention of stroke in patients with atrial fibrillation hepatic impairment.

Various chemical syntheses of apixaban have been reported in the literature.<sup>14-17</sup> Among them, the most common synthesis is that reported by Bristol Mayor Squibb, an innovator of apixaban.<sup>6</sup> The innovator route involves the use of p-amino iodobenzene as a key starting material (Scheme 1, A). The key step in the synthesis of apixaban involves C-N bond formation using Cu as a catalyst followed by ammonolysis to obtain apixaban. This route of synthesis of apixaban suffers from the disadvantages of (a) use of costly p-amino iodobenzene, (b) a C-N coupling reaction involving a costly metal catalyst, and (c) poor overall yield and thus has limitations of commercial viability. A modified synthesis of apixaban was reported that involved the use of p-amino nitrobenzene as a key starting material instead of p-iodo nitrobenzene (Scheme 1, B).<sup>14</sup> The key advantages of this route are its low cost and catalyst-free condition for C-N bond formation.

Process-related impurities are the impurities observed in drug substances and get generated in a manufacturing process.<sup>18-20</sup> The investigation of any process-related impurities in drug substances is a crucial aspect of API process research and development.<sup>21-23</sup> Knowing the chemical structure of process related impurities is important for assessing their toxicological nature and understanding the mechanism of impurity formations.<sup>24,25</sup> This information helps process scientists to categorize impurities according to the ICH classification, which further helps to define the limit of the impurity and its control strategy.<sup>26,27</sup> The relevant literature on this topic includes work by Jian *et al.*,<sup>28</sup> wherein they described the synthesis of impurity and its application for the treatment of coronary heart disease. During the manuscript preparation/submission, another literature on Apixaban was published by Ganta *et al.*,<sup>29</sup> wherein they described the development of an analytical method for the detection of GTI's in Apixaban. This work presents the isolation and characterization of process-related impurity observed during the preparation of apixaban by a modified process. Root cause investigation

is carried out to understand the factors responsible for the formation of impurity. Further, genotoxic assessment of impurity is also carried out to understand its toxicological nature. This work would be helpful to the pharmaceutical scientist working on the development of apixaban as an active pharmaceutical ingredient.

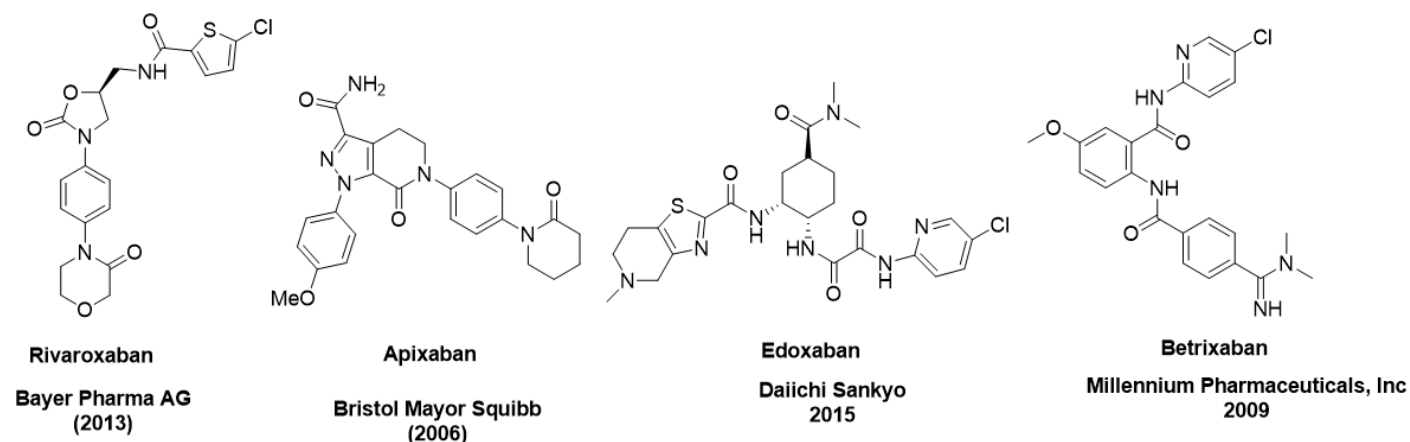
## MATERIALS AND METHODS

All the reagents and solvents required for the synthesis were obtained from commercial vendors and had the desired purity. NMR spectra of the synthesized compounds were recorded on Bruker 400 MHz spectrometers with TMS as the internal standard. Chemical shift is represented with  $\delta$  in parts per million. Mass spectra were recorded with a XEVO-TQSmicro#QEE0233 LC Mass Spectrometer. The IR spectra of synthesized compounds were recorded on Perkin Elmer Spectrum IR ES Version 10.6.0 by using the KBr pellet method. The purity of apixaban and its intermediates was determined by HPLC using an area normalization method.

## RESULTS

### 5-chloro-N-(4-nitrophenyl) pentanamide (APN-01)

2 L of clean and dry round-bottom flask was placed in a water bath. About 800 g of Tetrahydrofuran (THF) was charged into the flask, followed by P-nitroaniline (150 g) at 25-30°C under nitrogen atmosphere. The suspension was stirred for 10-15 min, and then the NaOH flakes (52.10 g) were charged at 25-30°C under a nitrogen atmosphere. The reaction mixture was cooled to -5 to 0°C. 5-Chlorovaleroylchloride (219.0 g) was slowly added over a period of 2-3 hr, and the reaction mass was maintained for 1-2 hr at -5 to 0°C. The progress of the reaction was monitored by HPLC. After completion of the reaction, the THF was distilled under vacuum below 65°C. The mixture was charged with water (750 g) at 25-30°C to obtain the suspension. The suspension was stirred for 1 hr, after which the suspension was filtered. The material was dried in a hot air oven for 5-6 hr to obtain dried



**Figure 1:** 2D Structures of rivaroxaban, apixaban, edoxaban, and betrixaban, the oral factor Xa inhibitors.

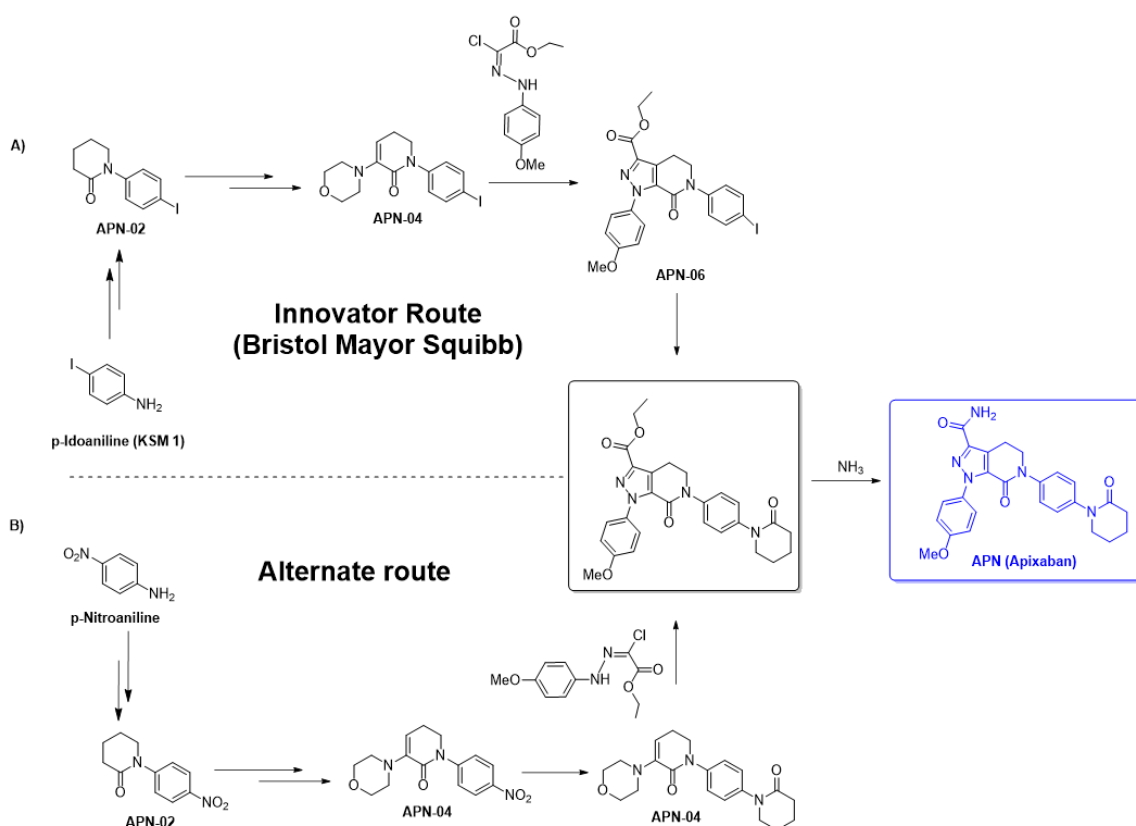
APN-01 (235.0 g, 85%).  $^1\text{H NMR}$  (400 MHz,  $\text{DMSO-}d_6$ ),  $\delta$ : 10.54 (s, 1H), 8.22 (d,  $J = 8.0$  Hz, 2H), 7.85 (d,  $J = 8.0$  Hz, 2H), 3.67 (t,  $J = 8$  Hz, 2H), 2.42 (t,  $J = 8.0$ , 2H), 1.72-1.78 (m, 4H);  $^{13}\text{C NMR}$  (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 171.8, 145.4, 141.9, 124.9, 118.5, 45.0, 35.5, 31.5, 22.1; IR (KBr,  $\text{cm}^{-1}$ ): 3300.58 (-NH), 1681.18 (-C=O), 1329.14 (-C-N); ESI-MS ( $m/z$ ) Calculated for  $\text{C}_{11}\text{H}_{13}\text{N}_2\text{O}_3\text{Cl}$ , 256.0; found 257.0 (M+H) $^+$ .

### 1-(4-nitrophenyl) piperidin-2-one (APN-02)

APN-01 (235.0 g) was dissolved in DMSO (800 gm), and  $\text{K}_2\text{CO}_3$  (191.20 kg, 1.4 mole equiv.) was charged. The temperature of the reaction mass was increased to 80-85°C and maintained for 6-8 hr. The progress of the reaction was monitored by HPLC. The reaction mass was cooled to 25-30°C. Approximately 1200 mL of water was taken in another RBF and cooled to 0-5°C. The reaction mixture was poured into cold water, and the resulting suspension was stirred for 1-2 hr. The reaction mixture was filtered, and the material was dried in a hot air oven at 50-60°C to obtain dried APN-02 (184.0 g, 70%).  $^1\text{H NMR}$  (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 8.24 (d,  $J = 8.8$  Hz, 2H), 7.63 (d,  $J = 8.8$  Hz, 2H), 3.71 (t,  $J = 5.8$  Hz, 2H), 2.46 (t,  $J = 8.0$  Hz, 2H), 1.83-1.91 (m, 4H, m);  $^{13}\text{C NMR}$  (100 MHz,  $\text{DMSO-}d_6$ )  $\delta$ : 169.5, 149.3, 144.1, 125.9, 123.8, 50.0, 32.8, 22.7, 20.6; IR (KBr,  $\text{cm}^{-1}$ ): 2951.41 (Ar-C-H), 1656.75 (-C=O), 1343.51 (-C-N); ESI-MS ( $m/z$ ) calculated for  $\text{C}_{11}\text{H}_{12}\text{N}_2\text{O}_3$ , 220.08; found 221.00 (M+H) $^+$ .

### 3-morpholino-1-(4-nitrophenyl)-5,6-dihydropyridin-2(1H)-one (APN-04)

A clean and dried four-neck RBF was taken. Chloroform (1500 g) and APN-02 (184.0 g) were added to RBF, and the reaction mixture was stirred. The reaction mixture was allowed to cool to 10-15°C.  $\text{PCl}_5$  was added to the reaction mass in a lot wise manner. The reaction mixture was heated to 55-60°C and maintained for 2-3 hr. The reaction mixture was allowed to cool to 20-30°C. Another RBF was arranged with water (3000 g) and cooled to 0-5°C. The reaction mixture was poured into RBF containing pre-chilled water. The reaction mixture was stirred at 25-30°C for 1 hr. The organic layer was separated by a separatory funnel. The organic layer was washed with water (40 mL). The organic layer was distilled to obtain crude APN-03. Morpholine (1200 g) was charged at 25-30°C to the RBF containing crude APN-03. The reaction mixture was heated to 120-130°C and maintained for 2-4 hr. The progress of the reaction was monitored by HPLC. Approximately 50% of the morpholine was distilled out under vacuum below 95°C. Water (1200 g) was slowly added to the reaction mixture over a period of 2-3 hr at 90-95°C. The reaction mixture was stirred for 1-2 hr, after which the reaction mixture was allowed to cool to 10-15°C. The reaction mixture was filtered through a Buchner funnel. The wet material was purified using methanol (270 g) to obtain pure APN-04. The material was dried in a hot air oven at 50-55°C to get dry APN-04 (141.0 g, 60%).  $^1\text{H}$



**Scheme 1:** Commercial route of syntheses of Apixaban; (A) Innovator route; (B) Alternate route.

NMR (400 MHz, DMSO- $d_6$ )  $\delta$ : 8.25 (d,  $J$  = 8.9 Hz, 2H), 7.60 (d,  $J$  = 8.9 Hz, 2H), 5.70 (t,  $J$  = 4.1 Hz, 1H), 3.84 (t,  $J$  = 8.0 Hz, 2H), 3.62 (t,  $J$  = 4.0 Hz, 4H), 2.76 (t,  $J$  = 4.0 Hz, 4H), 2.44-2.48 (m, 2H);  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 160.6, 148.7, 143.7, 142.4, 125.2, 123.8, 116.4, 65.9, 50.0, 49.0, 22.7; IR (KBr,  $\text{cm}^{-1}$ ): 2824.96 (Ar-C-H), 1671.12 (-C=O), 1505.88 (-C-O), 1214.19 (C-N), 1117.91 (C-N); ESI-MS ( $m/z$ ) calculated for  $\text{C}_{15}\text{H}_{17}\text{N}_3\text{O}_4$  303.12; found 304.00 (M+H) $^+$ .

### 1-(4-aminophenyl)-3-morpholino-5,6-dihydropyridin-2(1H)-one (APN-05)

APN-04 (141 g) was dissolved in methanol (3000 mL) and charged in a clean and dry autoclave. Raney nickel (1.41 g, 1% w/w) was charged to the reaction mass, and then autoclave was closed. A hydrogen pressure of 4-6  $\text{kg}/\text{cm}^2$  and a temperature of 55-60°C were applied. The reaction was maintained for 10-12 hr, and the progress of the reaction was monitored by HPLC. After the reaction was complete, the reaction mass was filtered through a hyflo bed. Methanol was partially distilled below 60°C to obtain a suspension. The suspension was cooled to 0-5 °C, filtered, and dried to get APN-05 (102 g, 80%).  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$ : 6.91 (d,  $J$  = 8.4 Hz, 2H), 6.52 (d,  $J$  = 8.4 Hz, 2H), 5.61 (t,  $J$  = 4.0 Hz, 1H), 5.06 (s, 2H), 3.63 (t,  $J$  = 4.0 Hz, 4H), 3.56 (t,  $J$  = 8.0 Hz, 2H), 2.77 (t,  $J$  = 4.0 Hz, 4H), 2.37 (q,  $J$  = 4.0 Hz, 2H);  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 160.6, 146.7, 143.1, 131.9, 126.3, 113.7, 113.5, 65.9, 49.9, 48.9, 22.9; IR (KBr,  $\text{cm}^{-1}$ ): 3425.59 and 3346.56 ( $\text{NH}_2$ ), 2804.84 (Ar-CH), 1613.65 (C=O), 1515.95 (-C-O), 1221.37 (C-N), 1120.79 (C-N); ESI-MS:  $m/z$  calculated for  $\text{C}_{15}\text{H}_{19}\text{N}_3\text{O}_2$  273.15; found 274.29 (M+1) $^+$ .

### 3-morpholino-1-(4-(2-oxopiperidin-1-yl) phenyl)-5,6-dihydropyridin-2(1H)-one (APN-07)

Dried and cleaned four-neck RBF was arranged with a water bath. APN-05 (102 g) was charged onto the RBF and dissolved in the MDC (1300 g). The reaction mixture was stirred for 10-15 min at 25-30°C. Triethylamine (76 gm, 2.0 mole equiv.) was slowly added to the reaction mixture over a period of 10-15 min., followed by the slow addition of 5-chlorovaleroyl chloride (69.5 g, 1.20 mole equiv.) at 25-30°C. The reaction mixture was stirred and maintained for 2-3 hr. The progress of the reaction was monitored by HPLC. Water (200 g) was charged to the reaction mixture, and the mixture was stirred for 20-30 min. The reaction mixture was then transferred to a separatory funnel. The layers were separated. The organic layer containing APN-06 proceeded *in situ* to the next stage. Next, the organic layer containing APN-06 was charged into another RBF, and KOH (41.66 gm, 2.0 mole equiv.) was charged. The temperature of the reaction mixture was raised to 40°C, and the reaction mass was maintained for 2-4 hr. The progress of the reaction was monitored by HPLC. After the

reaction was completed, MDC was distilled and degassed to get crude residue. The crude residue was charged with DM water (600 g), and the reaction mixture was stirred at 25-30°C for 1-2 hr. The suspension was filtered to obtain wet material. Wet material was dried in a hot air oven at 55-65 °C to get APN-07 (109.20 g, 77%).  $^1\text{H}$  NMR (400 M Hz, DMSO- $d_6$ )  $\delta$ : 7.35 (d,  $J$  = 8.5 Hz, 2H), 7.26 (d,  $J$  = 8.5 Hz, 2H), 5.70 (t,  $J$  = 4.3 Hz, 1H), 3.71 (t,  $J$  = 8.0 Hz, 2H), 3.64 (t,  $J$  = 4.0 Hz, 4H), 3.59 (t,  $J$  = 4.0 Hz, 2H), 2.78 (t,  $J$  = 4.0 Hz, 4H), 2.44 (q,  $J$  = 4.0 Hz, 2H), 2.38 (t,  $J$  = 8.0 Hz, 2H), 1.79-1.89 (m, 4H);  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 168.8, 160.5, 142.8, 140.9, 140.7, 126.2, 125.6, 114.8, 65.9, 50.8, 49.9, 48.3, 32.5, 23.0, 22.8, 20.8; IR (KBr,  $\text{cm}^{-1}$ ): 2964.34 (Ar-C-H), 1646.70 (-C=O), 1525.10 (-C=O), 1514.50 (-C-O), 1311.90 (-C-N), 1214.14 (C-N), 1114, 1070, 1050 (C-O stretching). ESI-MS  $m/z$  calculated for  $\text{C}_{20}\text{H}_{25}\text{N}_3\text{O}_3$  355.19, found 356.31 (M+H) $^+$ .

### Ethyl 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl) phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c] pyridine-3-carboxylate (APN-10)

Dried and clean four-neck RBF was arranged. APN-07 (100 g) and toluene (562 g) were charged onto the RBF. APN-08 (87.02 gm, 1.20 mole equiv.) was then charged onto the reaction mass, followed by the addition of triethylamine (71.2 g, 2.50 mole equiv.). The reaction mixture was stirred for 10-15 min at 25-30°C, after which the temperature of the reaction mass increased to 100-105°C. The reaction mass was maintained for 5-6 h, and the progress of the reaction was monitored by HPLC. Aq. HCl solution was added to the reaction mass at 25-30°C over a period of 1-2 hr. The reaction mixture was stirred and maintained for 4-6 hr. The progress of the reaction was monitored by HPLC. After the reaction is completed, toluene (350 g) was added to the reaction mixture. The temperature of the reaction mixture was increased to 70-80°C. The reaction mixture was stirred and maintained for 1 hr, after which layers were separated. The toluene layer was partially distilled under vacuum at 55-60°C. The reaction mixture was then cooled to 10-15°C. Then, the mixture was stirred for 60 min. The reaction mixture was filtered, and the wet cake was washed with prechilled toluene (50 g). The wet material was dried in a hot air oven at 55-60°C to obtain APN-10 (113.0 g, 82.70%).  $^1\text{H}$  NMR (400M Hz, DMSO- $d_6$ )  $\delta$ : 7.49 (d,  $J$  = 8.8Hz, 2H), 7.47 (d,  $J$  = 8.8Hz, 2H), 7.35 (d,  $J$  = 8.8Hz, 2H), 7.01 (d,  $J$  = 8.8Hz, 2H), 4.35 (q,  $J$  = 8.0 Hz, 2H), 4.07 (t,  $J$  = 8.0 Hz, 2H), 3.80 (s, 3H), 3.59 (t,  $J$  = 8.0 Hz, 2H), 3.20 (t,  $J$  = 8.0 Hz, 2H), 2.38 (t,  $J$  = 8.0 Hz, 2H), 1.79-1.88 (m, 4H), 1.33 (t,  $J$  = 8.0 Hz, 3H),  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ )  $\delta$ : 168.8, 161.3, 159.3, 156.3, 141.4, 139.6, 138.4, 133.0, 132.4, 126.7, 126.7, 126.3, 126.0, 113.4, 60.6, 55.4, 50.8, 50.7, 32.5, 22.9, 21.1, 20.8, 14.1; IR (KBr,  $\text{cm}^{-1}$ ): 2941.35 (Ar-C-H), 1737.22 (C=N), 1671.12 (C=O), 1651.01 (-C=O), 1515.94 (-C=N), 1467.08 (-C-O), 1250.11 (-C-N), 1142.34 (-C-N). (ESI-MS)  $m/z$  calculate for  $\text{C}_{27}\text{H}_{28}\text{N}_4\text{O}_5$  488.21, found 489.00 (M+H) $^+$ .

### 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide (Apixaban)

2 L clean and dry autoclave reactor was arranged. APN-10 (110 g) and methanol (2178 g) were charged into the autoclave, after which the autoclave was closed. Ammonia gas was purged into the reactor (4-6 kg/cm<sup>2</sup>) and then the temperature was increased to 70-75°C. The reaction mixture was maintained for 6-8 hr. The progress of the reaction was monitored by HPLC. After the reaction was complete on HPLC, the reaction mass was filtered through a hyflo bed. Methanol was partially distilled to get a suspension, which upon filtration and drying yielded pure apixaban (83.0 g, 80%). <sup>1</sup>H NMR (400M Hz, DMSO-d<sub>6</sub>) δ: 7.73 (br s, 1H), 7.51 (d, J = 8.0 Hz, 2H), 7.45 (br s, 1H), 7.35 (d, J = 6.4 Hz, 2H), 7.28 (d, J = 8.0 Hz, 2H), 7.00 (d, J = 8.0 Hz, 2H), 4.04 (t, J = 4.0 Hz, 2H), 3.79 (s, 3H), 3.58 (t, J = 8.0 Hz, 2H), 3.20 (t, J = 4.0 Hz, 2H), 2.38 (t, J = 4.0 Hz, 2H), 1.79-1.88 (m, 4H), <sup>13</sup>C NMR (100 MHz, DMSO-d<sub>6</sub>) δ: 168.8, 163.2, 159.1, 156.6, 141.4, 141.3, 139.7, 132.9, 132.5, 126.8, 126.3, 125.9, 125.2, 113.3, 55.4, 50.9, 50.8, 32.5, 23.0, 21.0, 20.9; IR (KBr, cm<sup>-1</sup>): 3300.04 (N-H), 2866.47 (Ar-C-H), 1682.59 (C=O), 1630.14 (C=O), 1595.08 (-C=O), 1519.24 (-C=N), 1343.85 (C-N), 1310.09 (-C-N). M/S m/z calculated for m/z C<sub>25</sub>H<sub>25</sub>N<sub>5</sub>O<sub>4</sub> 459.19, found 460.16 (M+H)<sup>+</sup>.

### 1-(4-aminophenyl)-3-morpholinopiperidin-2-one (APN-05 impurity)

<sup>1</sup>H NMR (400M Hz, DMF-d<sub>7</sub>) δ: 6.91 (d, J = 8.0 Hz, 2H), 6.66 (d, J = 8.4 Hz, 2H), 5.15 (s, 2H), 3.57-3.63 (m, 4H), 3.49-3.55 (m, 2H),

3.47-3.52 (m, 2H), 3.21 (t, 1H), 2.62-2.66 (m, 2H), 2.91-2.93 (m, 2H), 1.85-2.01 (m, 4H); <sup>13</sup>C NMR (100 MHz, DMF-d<sub>7</sub>) δ: 169.51, 147.76, 133.21, 127.36, 114.24, 67.65, 65.09, 51.53, 50.47, 25.68, 21.94; IR (KBr, cm<sup>-1</sup>): 3398.29 (-NH<sub>2</sub>), 1632.33 (C=O stretching), 1514.50 (C-O stretching), 1398.11 (C-N stretching). ESI-MS: m/z calculated for C<sub>15</sub>H<sub>21</sub>N<sub>3</sub>O<sub>2</sub> 275.16; found 276.21 (M+1)<sup>+</sup>.

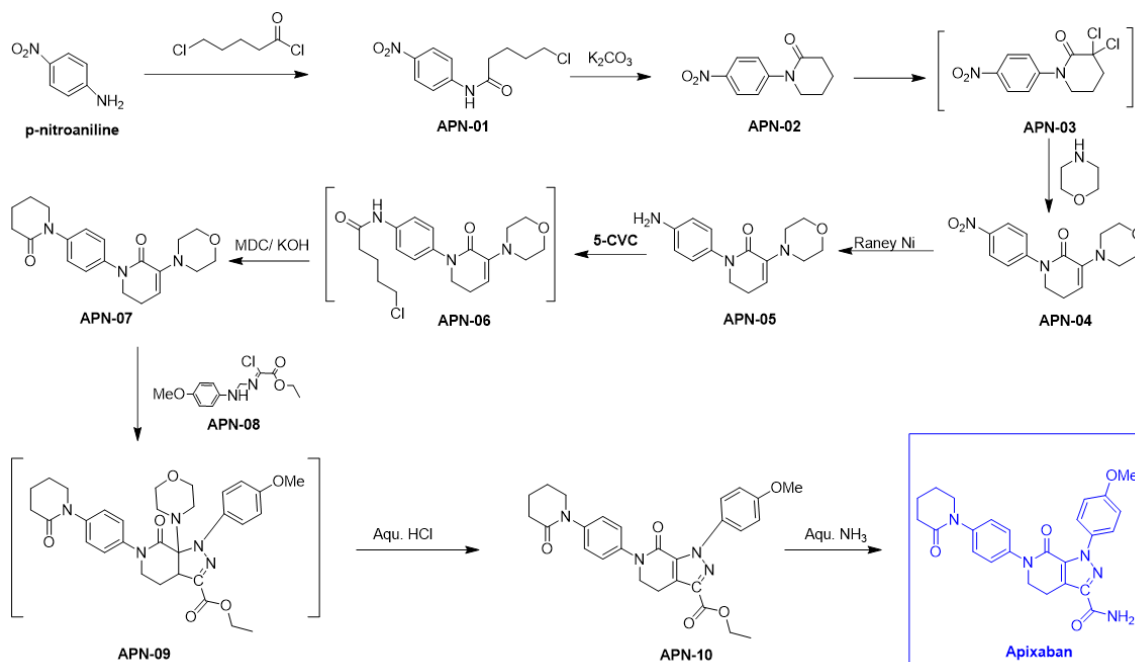
## DISCUSSION

### Apixaban synthesis

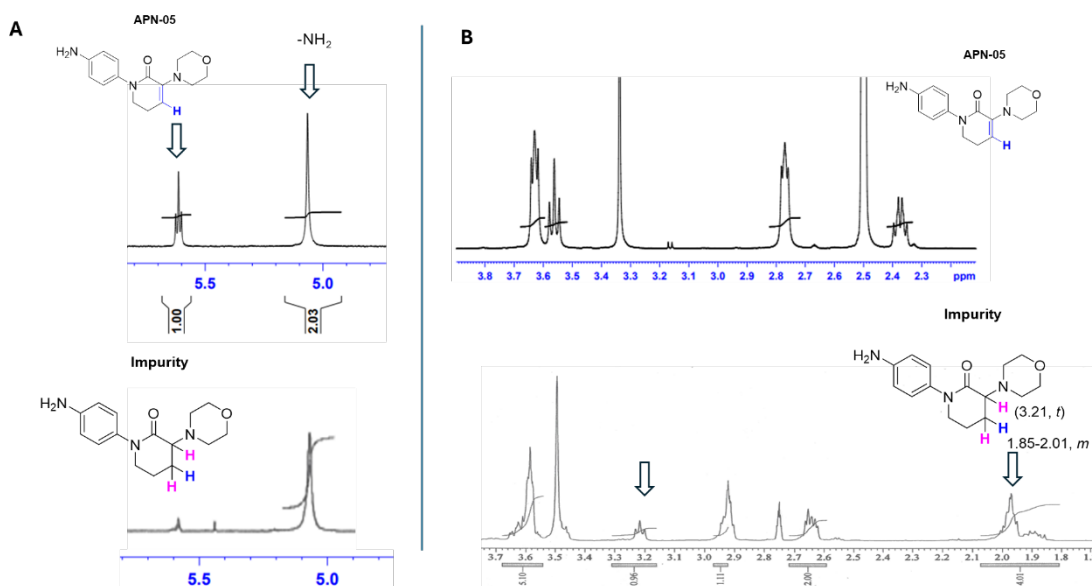
We have developed a process for the preparation of apixaban by slight modification of a literature known process. The optimized route of synthesis of apixaban consists of eleven chemical transformations (Scheme 2). The synthesis starts with the acylation of p-nitroaniline with 5-chlorovaleryl chloride followed by intramolecular cyclization to obtain APN-02. Chlorination of APN-02 using PCl<sub>5</sub> followed by reaction with morpholine led to the formation of APN-04. A reduction of APN-04 using Raney nickel led to the formation of APN-05. APN-05 again undergoes acylation and intramolecular cyclization to form APN-07, which is an intermediate required for the synthesis of apixaban. Finally, APN-07 and APN-08 react with each other to form APN-09 as an *in situ* intermediate, which leads to the formation of apixaban upon ammonolysis.

### Identification of unknown impurity

During the preparation of APN-05 from APN-04 by an optimized process (Scheme 2), an unknown impurity was observed at 0.30 RRT in the HPLC chromatograph of reaction monitoring samples. In all the R & D batches, this impurity was observed up to a level of ~1.0%. However, during scale-up studies in the pilot



Scheme 2: Synthesis of Apixaban (this work) by slight modification of the literature process.



**Figure 2:** Apixaban intermediate (APN-05) and its corresponding impurity along with  $^1\text{H}$  NMR overlay. A) Overlay of vinylic region of  $^1\text{H}$  NMR spectrum to demonstrate the absence of vinylic proton in  $^1\text{H}$  NMR spectrum of impurity; B) Overlay of  $^1\text{H}$  NMR spectrum to demonstrate the appearance of two additional signals in aliphatic region (3.2 ppm and 1.85-2.01 ppm, highlighted with downward arrow) of  $^1\text{H}$  NMR spectrum of impurity.

plant, the same impurity was found to be present in a reaction mass upto  $\sim 11.0\%$ . Hence, we became interested to know the structure of impurity in order to understand the root cause of its formation and possible control strategy during scale up studies. The reaction monitoring data of APN-05 of R & D batches and pilot plant batches are provided in Table 1.

### Isolation of unknown impurity

Next, we focused on the isolation of the impurity for its characterization. We isolated the impurity by first dissolving crude APN V in water. The filtered mother liquor was analyzed, which indicated the higher percent of impurity. The mother liquor was evaporated to get residue, which was subjected to flash chromatography to get impurity with  $\sim 95\%$  purity. A pure sample of impurity was used for analysis by LC-MS spectrometry, IR,  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectroscopic techniques.

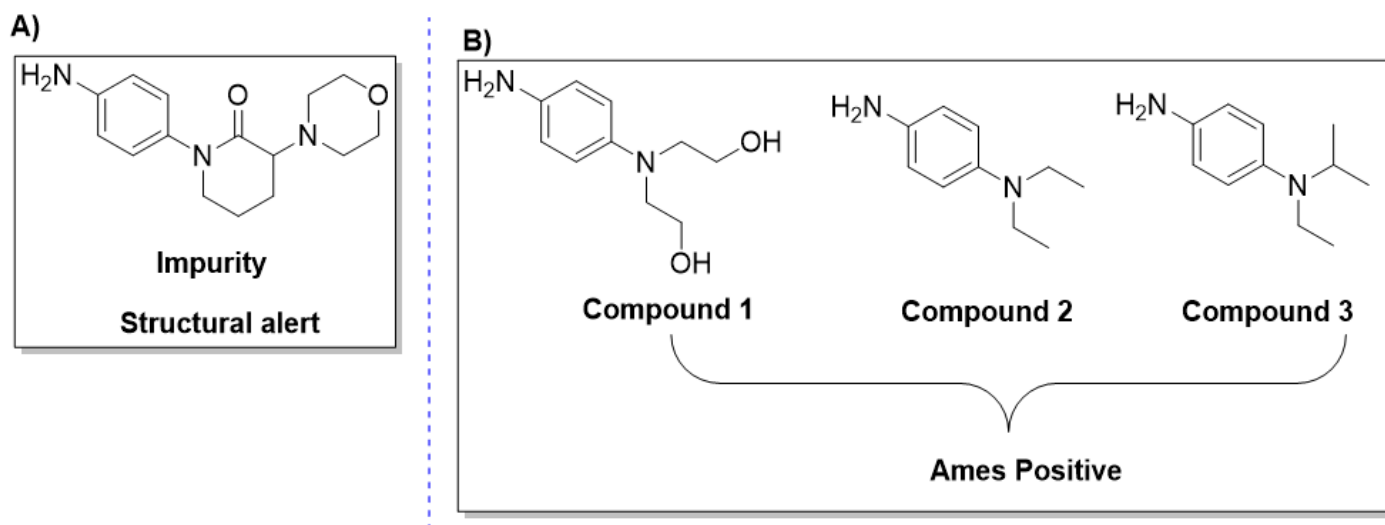
### Characterization of impurity

A systematic characterization study of impurity was carried out using spectrometric (LC-MS) and spectroscopic techniques ( $^1\text{H}$  and  $^{13}\text{C}$  NMR). The LC-MS analysis of impurity showed  $m/z$  276. The mass spectrum for parent compound i.e. APN-05 shows  $m/z$  274 correspond to  $(\text{M}+\text{H})^+$  ion peak. Thus, the mass spectrum of impurity suggests the presence of two additional proton in the structure of APN-05. The structural feature of APN-05 suggest that there is also a possibility of reduction of keto as well as double bond as a part of the 2-piperidone moiety (Figure 2). Then we analyzed the IR spectrum of an impurity. A stretching band at  $1632.33\text{ cm}^{-1}$  indicates the presence of a ketone functional

group. The IR spectrum rules out the possibility of reduction of the ketone moiety and hints toward the possibility of reduction of the double bond, which is part of the 2-piperidone moiety.

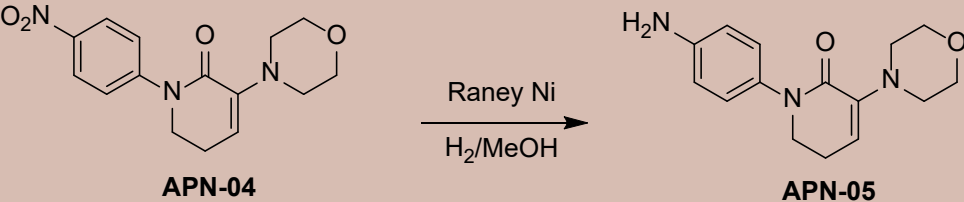
Next, we analyzed the  $^1\text{H}$  NMR spectrum of the impurity and compared it with the parent compound, i.e. APN-05. The  $^1\text{H}$  NMR spectrum of APN 05 showed a triplet at 5.61 ppm due to the presence of one vinylic proton ( $-\text{C}=\text{C}-\text{H}$ ) that is coupled with two protons present at the adjacent carbon. In the  $^1\text{H}$  NMR spectrum of the impurity, this signal was found to be absent, and an additional triplet of one proton was observed at 3.2 ppm. This pattern of  $^1\text{H}$  NMR spectrum clearly suggests the reduction of the ring double bond of 5,6-dihydropyridin-2(1H)-one moiety of APN-05 leading to an impurity with a piperidin-2-one moiety. The rest of the  $^1\text{H}$  NMR pattern of impurity is the same as of APN-05.

The  $^{13}\text{C}$  NMR spectrum of impurity also suggests that the ring double bond of 5,6-dihydropyridin-2(1H)-one moiety of APN-05 is reduced, and thus only four signals in the aromatic region were observed against six signals due to six aromatic protons in APN-05. Out of six signals, four signals are due to aromatic ring carbon and two signals are due to double bond carbon of the 5,6-dihydropyridin-2(1H)-one moiety present in APN-05. And thus, in  $^{13}\text{C}$  NMR spectrum of impurity only four signals are observed due to the aromatic ring. The  $^{13}\text{C}$  NMR spectrum of the impurity showed a signal due to carbonyl carbon at 169.5 ppm thus rulling out the possibility of reduction of the carbonyl group. All the above data suggest that the impurity is a hydrogenated product of APN-05 having the proposed structure as shown in Figure 2.



**Figure 3:** Genotoxic assessment of unknown impurity using Multicase Software. A) Impurity as a structural alert predicted by MultiCase; B) Structurally similar analogue of impurity which are known mutagen with positive Ames test.

**Table 1:** Reaction monitoring profile of APN-04 to APN-05 reaction.



Batch No.	Source	Batch size	Reaction monitoring by HPLC (%) <sup>a</sup>		
			% Conversion to APN-05	Unreacted APN-04 (%)	Unknown impurity at 0.30 RRT (%)
001	R & D	10.0 g	96.70	0.70	0.88
002	R & D	10.0 g	93.46	0.65	0.98
003	R & D	10.0 g	94.30	0.65	1.0
004	R & D	10.0 g	96.22	0.72	0.88
005	R & D	10.0 g	96.72	0.74	0.88
006	R & D	10.0 g	92.89	0.67	1.0
007	R & D	10.0 g	93.78	0.65	1.0
011	Pilot plant	20 kg	93.03	0.02	4.37
012	Pilot plant	20 kg	86.69	0.15	11.43

a 3-4 kg/cm<sup>2</sup>H<sub>2</sub> pressure, 50-60 °C temperature, 4-6 hr.

### Root Cause investigation

In order to understand the root cause for the formation of unknown impurity, we have carried out control experiments by varying various process parameters such as pressure, temperature, and reaction time against standard reaction conditions. Table 2, Entry 1 shows reaction monitoring data under standard condition wherein an unknown impurity was found to be 0.84%. In the next batch (Entry 2) reaction is performed under hydrogen pressure 9-10 kg/cm<sup>2</sup> against 3-4 kg/cm<sup>2</sup>. Under this reaction

condition, the level of unknown impurity was 0.80%. Though the impurity under discussion did not increase under high pressure, another unknown polar impurity was found to be 2.14%, which is generally present to a level of ~1.0% under standard conditions. In the next batch (Entry 3), the reaction is maintained up to 12-14 hr against 4-6 hr. Under this condition the level of impurity was 1.02%. Further, we performed another batch (Entry 4) with a reaction temperature of 65-70°C. Under this condition, though the unknown impurity under consideration was not increased i.e.1.0%, another single highest impurity of ~2.27% was observed

**Table 2: Control experimental studies for root cause identification of unknown impurity.**

Entry	Deviation in parameter	Reaction monitoring by HPLC (%)			Any SHI
		Conversion to APN-05	Unreacted APN-04	Unknown impurity	
1	No deviation (Standard condition) <sup>a</sup>	96.80%	ND	0.84%	1.12%
2	H <sub>2</sub> Pressure: 9-10 kg/cm <sup>2</sup>	94.67%	0.40%	0.80%	2.16%
3	Reaction time: 12-14 h.	96.22%	0.72%	0.88%	0.36%
4	Reaction temperature: 65-70°C.	92.89%	0.67%	1.00%	2.27%
5	Moisture content: ~10%	92.12%	0.50%	4.32%	0.84%

<sup>a</sup>3-4 kg/cm<sup>2</sup>H<sub>2</sub> pressure, 50-60°C temperature, 4-6 hr.

in non-polar region, which led to the lower conversion of ~92.89%. Next, we performed a negative experiment (Entry 5) where 10% w/w water was doped to the reaction mass, and then the hydrogenation reaction was carried out. Surprisingly, an increased level of formation of unknown impurity i.e.~3.91% is observed against 0.88% under this condition. From this data, it can be concluded that moisture is the critical process parameter that is responsible for the formation of unknown impurity.

### Genotoxic Impurity (GTI) assessment of unknown impurity

After identification of the structure of the unknown impurity, we have carried out genotoxic assessment of the impurity using MultiCase software.<sup>30</sup> MultiCASE software is a computational toxicology platform that includes tools for predicting genotoxicity. Specifically, the software, including CASE Ultra, utilizes (Q)SAR models and structural alerts to assess the potential for bacterial mutagenicity/ICH M7, genotoxicity, and other toxicological endpoints. Based on the assessment report, the unknown impurity is predicted to be positive and thus is classified as ICH M7 class 3 compound, i.e. those that possess alerting structural features for mutagenicity but lack sufficient mutagenicity data to be classified as a class 2 or 1. There are other structurally similar analogues of impurity that are known mutagen/genotoxic based on the Ames test (Figure 3).

### CONCLUSION

This work demonstrates, the identification of new process-related impurity generated during the synthesis of Apixaban. The unknown process-related impurity was found to be generated during the preparation of APN-05 by reduction of APN-04 with Raney Ni/H<sub>2</sub> under 3-6 kg/cm<sup>2</sup> pressure at 50-60°C. The unknown impurity is present to a level of ~1.0% in R & D batches of 10.0 g scale, but during the scale up study at 20 kg scale, the impurity was found to be present upto a level of ~11.0%. The root cause for the formation of the impurity to a higher level in scale-up batches is attributed to the presence of moisture in the reaction mass. The unknown impurity was isolated by flash chromatography and

characterized by spectroscopic techniques such as <sup>1</sup>H NMR, <sup>13</sup>C NMR, IR, and ESI-MS spectrometry. Based on the spectral data, the unknown impurity was identified as 1-(4-aminophenyl)-3-morpholinopiperidin-2-one, which is a reduced form of APN-05. Further, this impurity is predicted as a structural alert by Multi Case and thus is classified as ICH M7 class 3 compound, i.e. compounds that possess a structural alert but the experimental evidence for their mutagenicity is not available.

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### CONFLICT OF INTEREST

There is no conflict of interest declared by the authors of the manuscript.

### ABBREVIATIONS

**LC:** Liquid Chromatography; **HPLC:** High Performance Liquid Chromatography; **KBr:** Potassium Bromide; **IR:** Infrared; **NMR:** Nuclear Magnetic Resonance; **NDA:** New Drug Application; **FDA:** Food and Drug Administration; **BMS:** Bristol Mayer Squibb; **VTE:** Venous Thromboembolism; **TMS:** Trimethylsilyl; **APN:** Apixaban; **NaOH:** Sodium Hydroxide; **DMSO:** Dimethylsulfoxide; **MDC:** Dichloromethane; **R & D:** Research and Development; **LC-MS:** Liquid Chromatography-Mass Spectrometry.

### SUMMARY

This study focuses on isolation and characterization of an unknown impurity observed during synthesis of Apixaban along with genotoxic assessment of the impurity. Identified impurity is a reduced form of APN-05. The proposed root cause for the formation of this impurity is presence of moisture in a reaction mass. Further, this impurity is predicted as structural alert by MultiCase and thus is classified as ICH M7 class 3 compound i.e.

compounds which possess structural alert but the experimental evidence for their mutagenicity is not available. This information would be of great use to scientists who are working in the area of process research and development of Active Pharmaceutical Ingredients (API).

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