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# LC-Q-TOF-MS driven identification of potential degradation impurities of venetoclax, mechanistic explanation on degradation pathway and establishment of a quantitative analytical assay method

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## Abstract

Venetoclax is a selective orally active Bcl-2 protein inhibitor very recently approved by USFDA to treat chronic lymphocytic leukemia and other hematological malignancies. Postmarketing surveillance of any drug depends on its acceptability based on risk to benefit ratio. When risk outweighs the benefits, withdrawal of an already marketed drug is warranted. Presence of impurity is the primary cause of increased risk in a drug substance or drug product. With the discovery of newer molecules, it is of great importance to establish advanced analytical techniques for quantification of the drugs as well as their related impurities to address the prospective regulatory queries even if it is already in the market. In this study, a quantitative analytical assay method has been developed and validated for quantification of venetoclax in presence of its degradation impurities. A stress study was performed to examine the stability of the drug in hydrolytic, oxidative, thermolytic and photolytic environments. Venetoclax was found to be prone to degradation in acidic hydrolytic and oxidative stress conditions. Three new degradation impurities have been identified and characterized with the help of LC-Q-TOF-MS with accurate mass measurement and their putative structures have been proposed. Furthermore, for the first time, a possible degradation pathway has been established with mechanistic explanation. Moreover, the analytical method developed in this study will be of immense help for routine analysis of quality control and stability study samples of venetoclax in industry and research laboratories.

**Keywords:** Venetoclax, Analytical assay method, Validation, Degradation product, Characterization, LC-Q-TOF-MS

## Introduction

Venetoclax (VEN) is a selective, novel, and orally active B-cell lymphoma 2 (Bcl-2) protein inhibitor very recently approved by the United States Food and Drug Administration (USFDA) to treat chronic lymphocytic leukemia and other hematological malignancies (Mato et al. 2018).

Chemically VEN belongs to biaryl acylsulfonamide class of anticancer molecule indicated for the treatment in adult cancer patients with 17p deletion (chromosome abnormality) or tumor suppression gene TP53 mutation or chemo-immunotherapy resistance. Overexpression of Bcl-2 protein results in the resistance of cancerous cells to apoptosis and hence, it is considered an important target for anticancer chemotherapy. VEN selectively binds to Bcl-2 protein and helps to restore apoptosis process by displacing proapoptotic proteins triggering permeability of outer membrane of mitochondria and

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the activation of caspases (Žigart and Časar 2019; Roberts et al. 2016).

Globally, researchers are engaged in a continuous effort to discover anticancer drugs with a much higher safety profile (Jain et al. 2019; Sharma et al. 2019). Post-marketing surveillance of any drugs depends on its acceptability based on risk to benefit ratio. When risk outweighs the benefits, withdrawal of an already marketed drug is warranted. Presence of impurity is the primary cause of increased risk in a drug substance or drug product. With the discovery of newer molecules, it is of great importance to establish advanced analytical techniques for quantification of the drugs as well as their related impurities to address the prospective regulatory queries even if it is already in the market (Sahu and Sengupta 2020; Sharma et al. 2020; Gousuddin et al. 2017). Forced degradation is a strategy employed to cause stress decomposition of a molecule to generate degradation impurities for their subsequent identification. It is carried out at conditions more severe than accelerated conditions, which further help in evaluating the stability of a molecule (Inturi et al. 2018; Blessy et al. 2014). Stability is the ability of drugs in bulk form and their pharmaceutical product to remain within the specifications to maintain its purity, strength, quality and identity throughout their retest or shelf-life period. Assessment of degradation impurities through stress testing is an obvious requirement in a drug development program that helps in understanding the degradation behavior of a drug (Brümmer 2011; Sengupta et al. 2018). It also provides physicochemical and stereochemical stability of bulk drug and its formulation. According to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and other regulatory guidelines, it is a mandatory requirement to perform stress studies and develop stability indicating assay method before filing the registration dossier (Inturi et al. 2018). This study also helps in selecting appropriate dosage form and improvement in the production and packaging process (Hotha et al. 2013; Bhavsar et al. 2016). Stress studies at the developmental stage give a database that helps to select an excipient for further formulation development, container and closure system selection and also modification in storage conditions (Bajaj et al. 2012; Alsante et al. 2007). As a whole, stress studies are considered an essential part of the drug development cycle. Among all the sophisticated techniques chromatographic retention assisted mass spectrometry including Q-TOF is considered an excellent tool in characterization of unknown drug-related impurities with high selectivity and sensitivity (Watkins 2020; Chang et al. 2018; Kitagawa 2019).

Various analytical techniques have been reported for quantification of VEN. Liu et al reported a high

performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) method for absorption and metabolism study of the drug (Liu et al. 2017). However, a literature search revealed that there is no analytical assay method reported for quantification of VEN in presence of its degradation impurities. Therefore, we aimed to perform forced degradation of VEN and development of a quantitative analytical method in presence of its degradation products. Moreover, we also aimed to identify and characterize the potential degradation impurities of VEN by LC-quadrupole-time of flight (Q-TOF)-MS analysis.

## Experimental

### Chemicals

VEN (purity 98.81 %) was procured from Clearsynth. Acetonitrile (ACN), hydrogen peroxide (30 %), and formic acid were obtained from Qualigens, Thermo Fischer Scientific. Ammonium formate was obtained from Sigma Aldrich. Sodium hydroxide (NaOH) was procured from Fischer scientific. Hydrochloric acid (HCl) was purchased from Merck. For buffer and sample preparation, ultrapure water from Milli Q system (Millipore, USA) was collected.

### Instrumentation

The HPLC system (Agilent) used for method development of VEN was comprising of binary pumps, column oven, autosampler, and diode array detector. OpenLab software was used for data collection and acquisition for report generation. LC-Q-TOF-MS system employed for characterizing the degradation impurities was comprising of an HPLC system (Agilent) connected to Q-TOF mass spectrometer (Agilent 6545 series) through an ESI interface ionization source. Mass Hunter software was employed for data processing and acquisition. Photolytic stress experiment was performed using a photostability chamber (Newtronic Life care). Hydrolytic degradation study was performed in a Radleys apparatus (Essex UK). A thermolytic stress study was conducted using a hot air oven (Heratherm, Thermo Scientific). For properly dissolving the sample, an ultra sonicator (Antech Scientific) was used. The pH meter of Eutech Instruments was employed for pH adjustment of stress samples and mobile phases.

### Chromatographic conditions

Liquid chromatographic separation was achieved through an Agilent Zorbax eclipse plus C18 column (250 X 4.6 mm; 5  $\mu$ m) using ammonium formate (10 mM, pH 5 adjusted with formic acid): ACN as mobile phase at ambient temperature and a flow rate of 1 mL/min. Mobile phase was freshly prepared each time before the analysis. All separations were done at ambient

temperature. The run time was optimized to 30 min in a gradient program ( $T_{\min}/\%ACN$ : 0/60, 3/60, 17/90, 20/90, 25/60, 30/60) for separation of VEN and its degradation products. The column was kept at 30°C and analytes were detected at a wavelength of 286 nm. A 10  $\mu$ L injection volume was sufficient for detection of VEN and its degradation products (DPs).

#### Preparation of drug solution

About 5 mg of VEN was weighed and transferred to a 10 mL volumetric flask to which 5 mL of ACN was added and sonicated for 5 min. Thereafter, the volume was made up to 10 mL with ACN to make a stock solution of about 500  $\mu$ g/mL. From this stock solution, 4 mL was diluted up to 10 mL with ACN to give a final working solution of about 400  $\mu$ g/mL, which was used for method development.

#### LC-MS conditions

The LC-MS/MS analysis of VEN was carried out in ESI positive mode. Fragmentor voltage was set at 130 V, capillary voltage at 3500 V, skimmer voltage at 64 V, collision energy at 30 eV, drying gas temperature at 320 °C (8 L/min), nebulising gas pressure at 35 psi, sheath gas temperature and flow at 350°C and 11 L/min, respectively. Nitrogen was used as drying, nebulizing and collision gas. The liquid chromatographic separation was done on Agilent Poroshell 120 SB C18 column by using ammonium formate (10 mM, pH 5 adjusted with formic acid): ACN as mobile phase at ambient temperature and 0.4 mL/min flow. A sample volume of 2  $\mu$ L was injected for each run.

#### Stress studies

Stress studies on VEN were performed by applying different stress conditions given in ICH Q1A (R2) guideline (ICH 2003). All the stress experiments were performed on 1 mg/mL solution of VEN. It was subjected to various stress conditions like hydrolysis, oxidation, thermal and photolytic degradation. Acidic, basic and neutral hydrolysis was performed with 2 N HCl, 2 N NaOH, and water, respectively at 70 °C for 72 hr. Oxidative degradation was performed by exposing the drug to 0.3 % hydrogen peroxide ( $H_2O_2$ ) at ambient conditions for 48

hr. For thermolytic stress, the drug was uniformly spread on a Petri plate and kept in a hot air oven at 80 °C for 7 days. Photolysis of VEN was carried out in a photostability chamber by keeping solid form of the drug to ultra-violet (UV) and fluorescence light with a minimum intensity of 200 Whr/m<sup>2</sup> and 1.2 million.lux.hr, respectively. The maximum UV and fluorescence light exposure was 400 Whr/m<sup>2</sup> and 6 million.Lux.hr, respectively. For photo-degradation, control samples covered with aluminum foil (to prevent the exposure of light) were also placed along with stress samples in the photostability chamber. All stress samples were compared with blank and control samples exposed to the same conditions. The optimized stress conditions applied in stress degradation of VEN are summarized in Table 1.

#### Generation of forced degraded samples

##### Acidic hydrolysis

Acidic hydrolytic stress sample was prepared by weighing about 5 mg of VEN and transferred to Radleys tube to which 5 mL of ACN was added and sonicated for 2 min. To this drug solution, 5 mL of 2 N HCl was added and final concentration of stress sample was 500  $\mu$ g/mL. A blank sample was also prepared by taking 5 mL of 2 N HCl and 5 mL of ACN to check if any interference is present due to the solvent used for sample preparation. Both sample and blank solutions were placed on Radleys apparatus with continuous stirring with a magnetic bead at 300 rpm and 70 °C for 72 hrs.

##### Basic hydrolysis

Basic hydrolytic stress sample was prepared by weighing 5 mg of VEN and transferred to Radleys tube to which 5 mL of ACN was added and sonicated for 2 min. To this drug solution, 5 mL of 2 N NaOH was added and final concentration of stress sample was 500  $\mu$ g/mL. A blank sample was also prepared by taking 5 mL of 2 N NaOH and 5 mL of ACN to check if any interference is present due to the solvent used for sample preparation. Both sample and blank solutions were placed on Radleys apparatus with continuous stirring with a magnetic bead at 300 rpm and 70 °C for 72 hrs.

**Table 1** Summary of degradation behaviour of VEN

Sr.no.	Degradation study	Conditions of stress study	% Drug degraded	Retention time of degradants (minutes)
1.	Acidic hydrolysis	2N HCl for 72hrs	7.31%	DP1 at 16.1 min
2.	Basic hydrolysis	2N NaOH for 72hrs	No degradation	-
3.	Neutral hydrolysis	Water for 72hrs	No degradation	-
4.	Oxidative degradation	0.3% $H_2O_2$ for 48hrs	44.46%	DP2 at 4.2 min and DP3 at 6.5 min.
5.	Thermal degradation	80°C for 7days	No degradation	-
6.	Photolytic degradation	UV and fluorescent light for 5 cycles	No degradation	-

### Neutral hydrolysis

Neutral hydrolytic stress sample was prepared by weighing 5 mg of VEN and transferred to Radleys tube to which 5 mL of ACN was added and sonicated for 2 min. To this drug solution, 5 mL of Milli Q water was added and final concentration of stress sample was 500  $\mu\text{g/mL}$ . A blank sample was also prepared by taking 5 mL of water and 5 mL of ACN to check if any interference is present due to the solvent used for sample preparation. Both sample and blank solutions were placed on Radleys apparatus with continuous stirring with a magnetic bead at 300 rpm and 70  $^{\circ}\text{C}$  for 72 hrs.

### Oxidative degradation

Oxidative stress sample was prepared by weighing 5 mg of VEN and transferred to 10 mL of volumetric flask to which 5 mL of ACN was added and sonicated for 2 min. To this drug solution, 5 mL of 0.3%  $\text{H}_2\text{O}_2$  was added and final concentration of stress sample was 500  $\mu\text{g/mL}$ . A blank sample was also prepared by taking 5 mL of

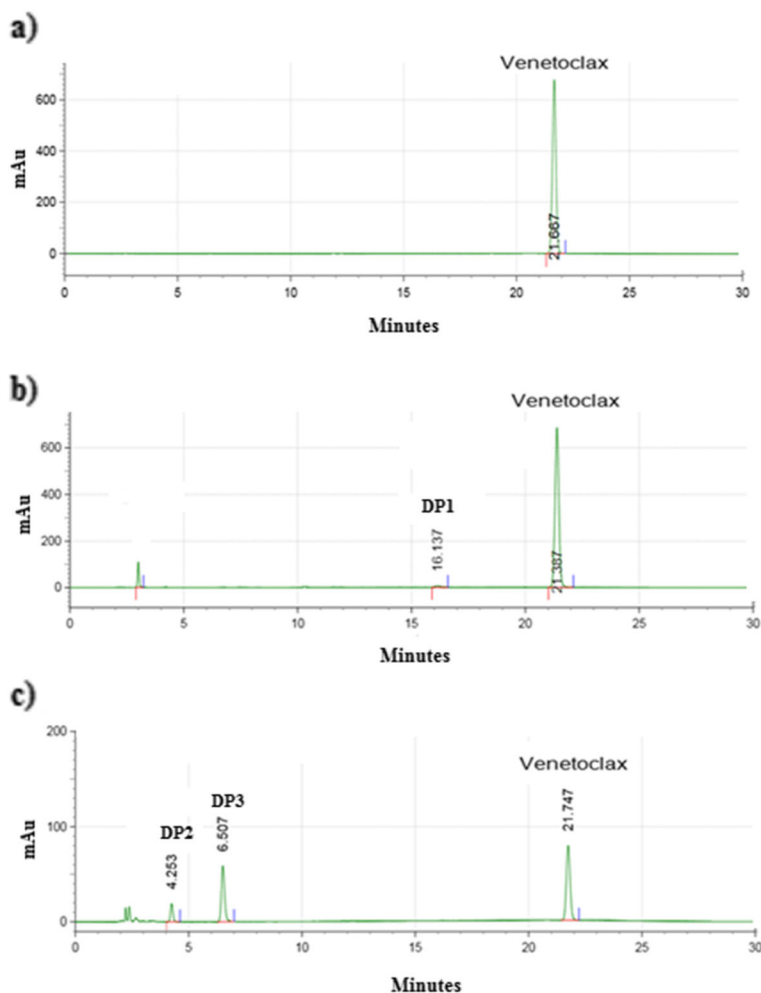
0.3%  $\text{H}_2\text{O}_2$  and 5 mL of ACN to check if any interference is present due to the solvent used for sample preparation. Both sample and blank volumetric flask are kept at room temperature in a dark place for 5 days.

### Thermal degradation

For thermal stress study, about 5 mg of VEN was taken and placed in a Petri plate and it was uniformly spread on the surface of Petri plate with the help of a spatula. After that, it was covered with a lid and kept in the hot air oven at 80  $^{\circ}\text{C}$  for 7 days.

### Photolytic degradation

For photolytic stress study, about 5 mg of VEN was taken and placed in Petri plates and it was uniformly spread on the surface of Petri plates with the help of a spatula. For solid control, 5 mg of drug was placed in a Petri plate and it was wrapped with aluminum foil. All Petri plates were placed in photostability chamber. Two Petri plates were kept in UV chamber, out of which one



**Fig. 1** HPLC chromatogram of a) Standard b) Acidic hydrolysis stressed c) Oxidative stressed sample

was control and the light exposure was from 200 to 400 wat.hr/m<sup>2</sup>. Another two Petri plates were kept in fluorescent chamber and exposed to 1.2 to 6 million.Lux.hr illumination.

#### Preparation of stress sample for analysis

ACN was used as a solvent for preparation of stress degraded samples. 1 mL of acid and base degradation sample was withdrawn at different time points and neutralized by 2 N NaOH and 2 N HCl, respectively with the aid of pH paper. Each sample after stress exposure was subjected to dilution for achieving a final concentration of 400 µg/mL. Samples were then filtered (0.22 µ membrane filter) and injected into the analytical systems.

#### Method validation

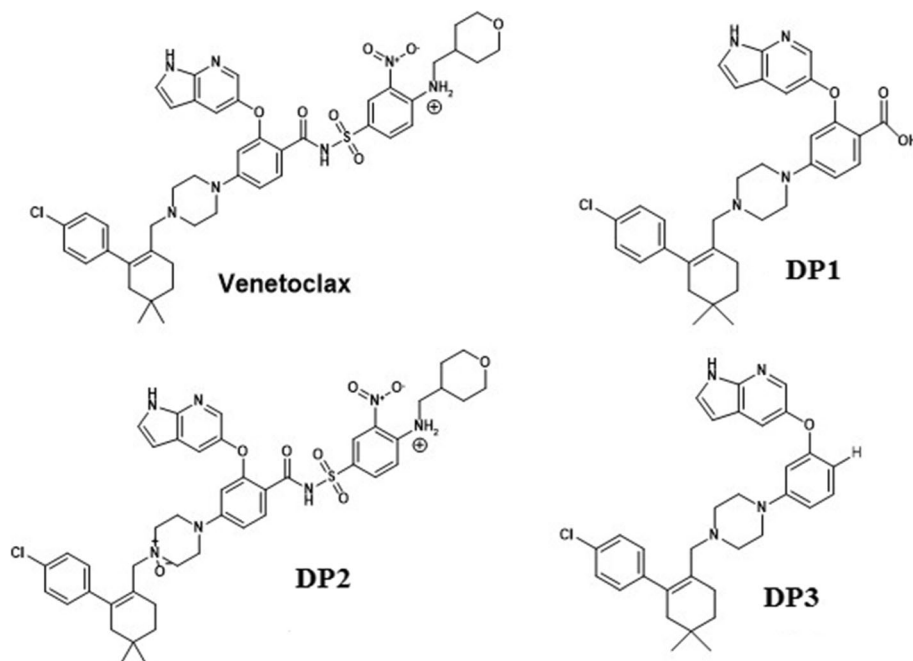
The developed assay method was validated according to the ICH Q 2 (R1) guidelines. The linearity of the method was established by taking seven calibration standards in triplicate (n=3) from 50 to 600 µg/mL. Intrabatch and inter-batch precision was evaluated by injecting six replicates of 400 µg/mL of one batch in a single day and twelve replicates of 400 µg/mL between two different batches in two different days, respectively. Accuracy of the method was determined in terms of recovery where three different concentration (80%, 100%, 120% of 400 µg/mL) of the drug was spiked (n=3) in the mixtures fortified with excipients. Then the % recovery was

calculated by comparing the area of spiked samples against standard samples.

## Results and discussions

#### Optimization of HPLC method

Preliminary chromatographic separations were carried out by using different columns, different mobile phases, at different pH and modifying different mobile phase ratios to resolve VEN and its degradation products, have good peak shape and symmetry. Initial runs were carried out in an isocratic mode to check the peak purity of VEN and to select the appropriate buffer system. Further trials were carried out in gradient mode on Waters Reliant C18 column (250 mm x 4.6 mm, 5 µm) using 10 mM ammonium acetate: ACN as a mobile phase. In this trial, poor peak shape, baseline noise and poor resolution were observed. Finally, the method was optimized by changing the column to Zorbax eclipse C18 column (250 mm x 4.6 mm, 5 µm) using ammonium formate (10 mM, pH 5 adjusted with formic acid): ACN as mobile phase at ambient temperature in a linear gradient program (T<sub>min</sub>/%ACN: 0/60, 3/60, 17/90, 20/90, 25/60, 30/60). The column was kept at 30 °C. At 286 nm wavelength, VEN and its DPs were detected and quantified well. An injection volume of 10 µL was selected for analyzing VEN and its DPs. Gradient program provided a better separation of all peaks as compared to isocratic program. By applying this chromatographic method, VEN and all degradation products were well separated with good peak shapes which are shown in Figure 1.



**Fig. 2** Structures of VEN and its DPs



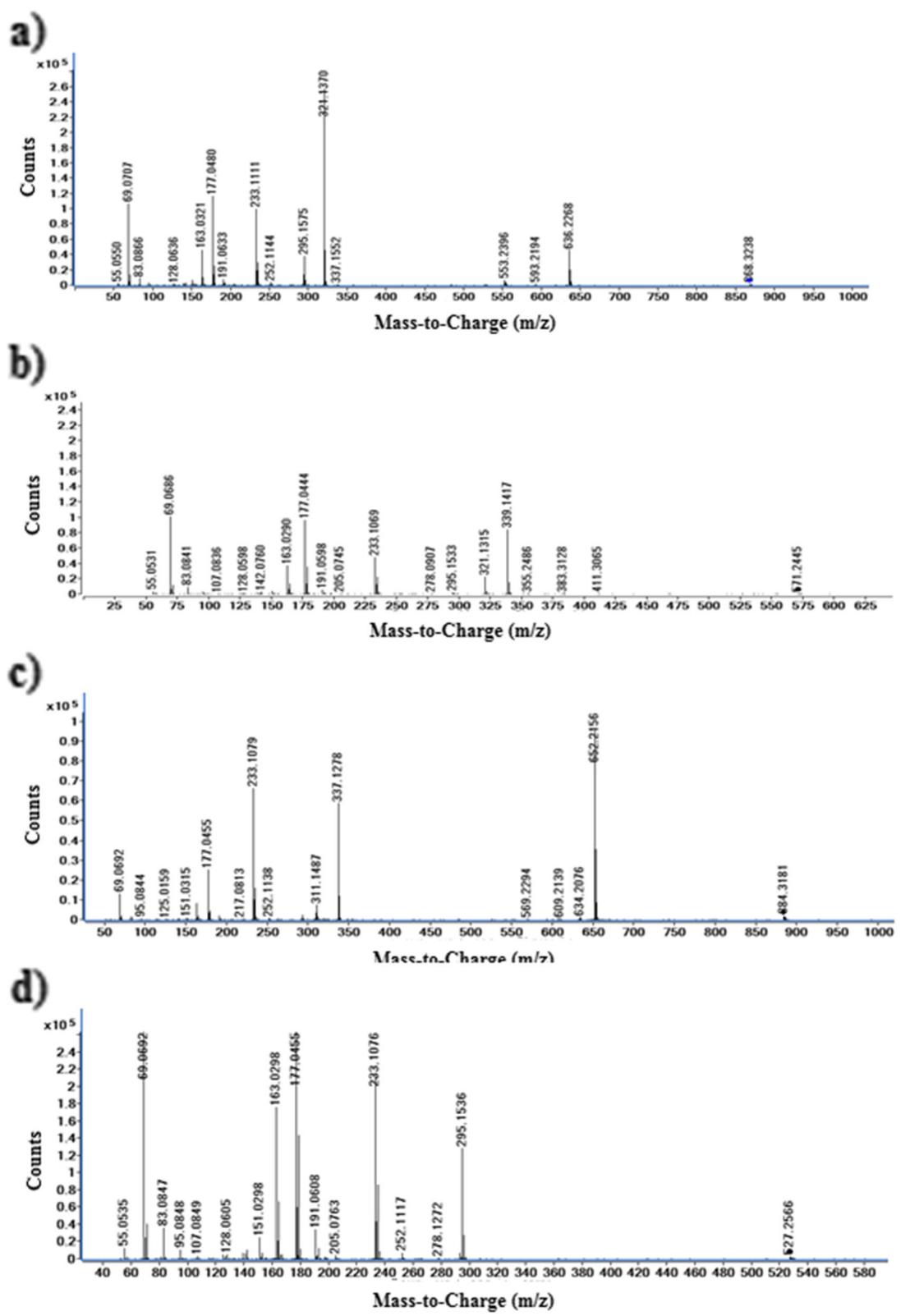


Fig. 3 LC-MS/MS spectra of a) VEN b) DP1 c) DP2 d) DP3

### Degradation pattern of VEN

The degradation pattern was studied by injecting the stressed samples to HPLC and further resolving the peaks of VEN and all DPs. VEN was degraded in acidic hydrolysis and oxidative stress. The drug was stable in basic hydrolysis, neutral hydrolysis, thermal and photolytic conditions. DP1 was formed in acidic hydrolysis. Moreover, two separate DPs (DP2 and DP3) were also observed in oxidative degradation.

### Structural characterization of VEN and its DPs

The degradation impurities generated in acidic hydrolysis and oxidative stress were subjected to mass spectrometric analysis. The  $m/z$  ratio and mass fragmentation patterns were studied for VEN and its DPs. The structures were proposed based on their accurate mass measurement and calculating ppm error. Structures of VEN and all its DPs are shown in Figure 2. Moreover, MS/MS spectrum of VEN and the DPs are shown in Figure 3.

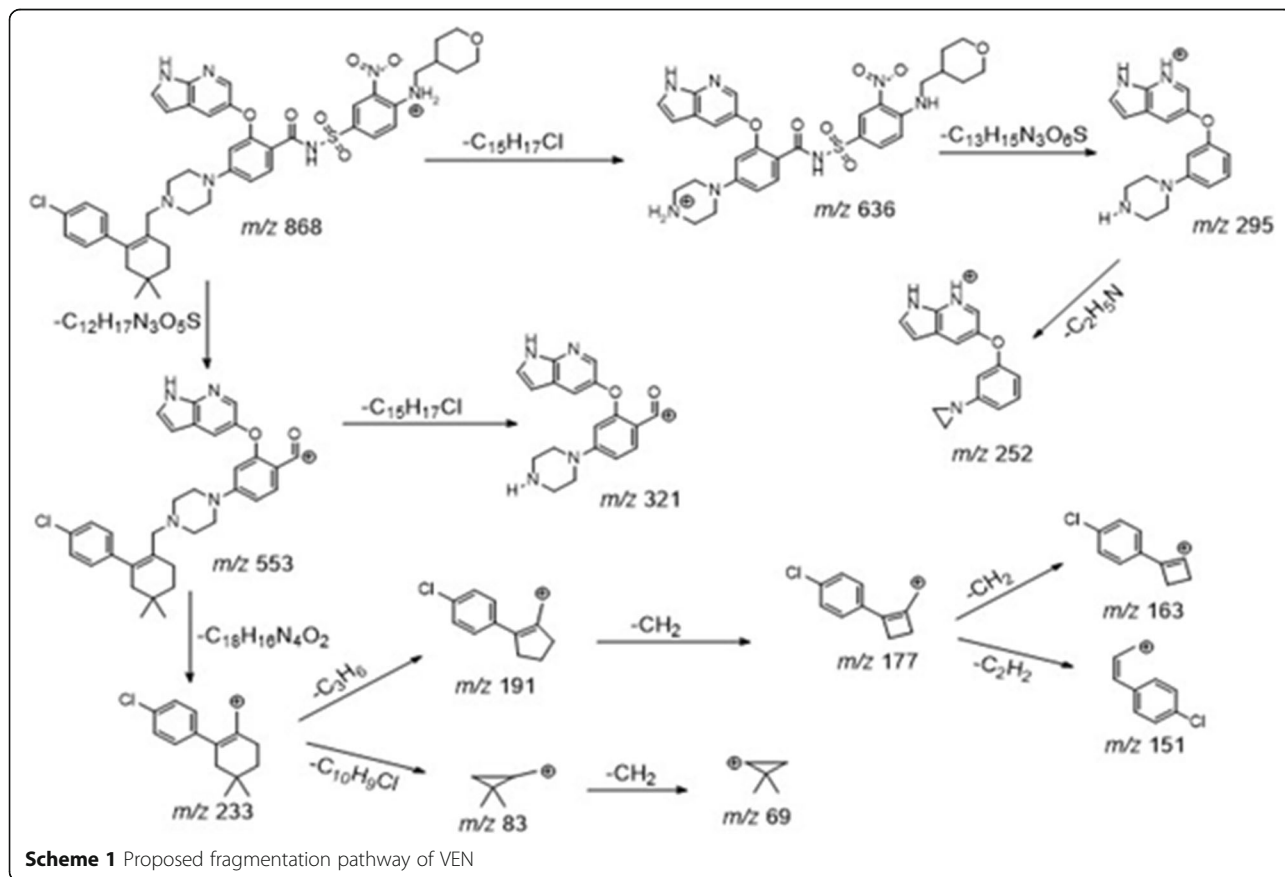
### MS/MS profiling of VEN

MS/MS spectrum of VEN (Figure 3a) showed protonated molecular ion at  $m/z$  868.3238 ( $C_{45}H_{51}ClN_7O_7S^+$ ) in positive ionization mode. Primary fragment ion formed at  $m/z$  636 (losing chlorophenyldimethyl-

cyclohexenyl moiety), 553 (losing benzene sulfonamide), 321 (losing chlorophenyldimethyl-cyclohexenyl moiety from  $m/z$  553), 295 (losing benzene sulfonamide moiety from  $m/z$  636), 233 (losing pyrrolopyridine-4-piperazine phenyl moiety from  $m/z$  553), 191 (losing cyclopropane moiety from  $m/z$  233), 177 (losing methylene group from  $m/z$  191), 163 (losing methylene group from  $m/z$  177). Moreover, additional fragments were also observed at  $m/z$  151, 83 and 69. The fragmentation patterns are shown in Scheme 1. The elemental composition was confirmed by accurate mass measurement as mentioned in Table 2.

### MS/MS profiling of DP1

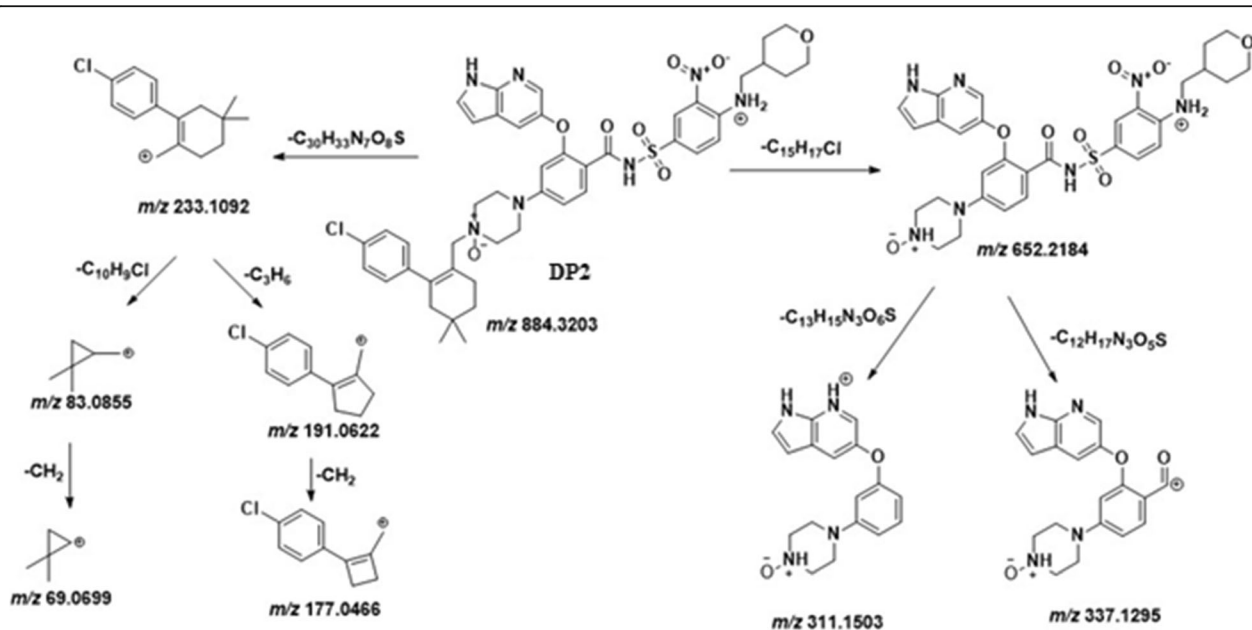
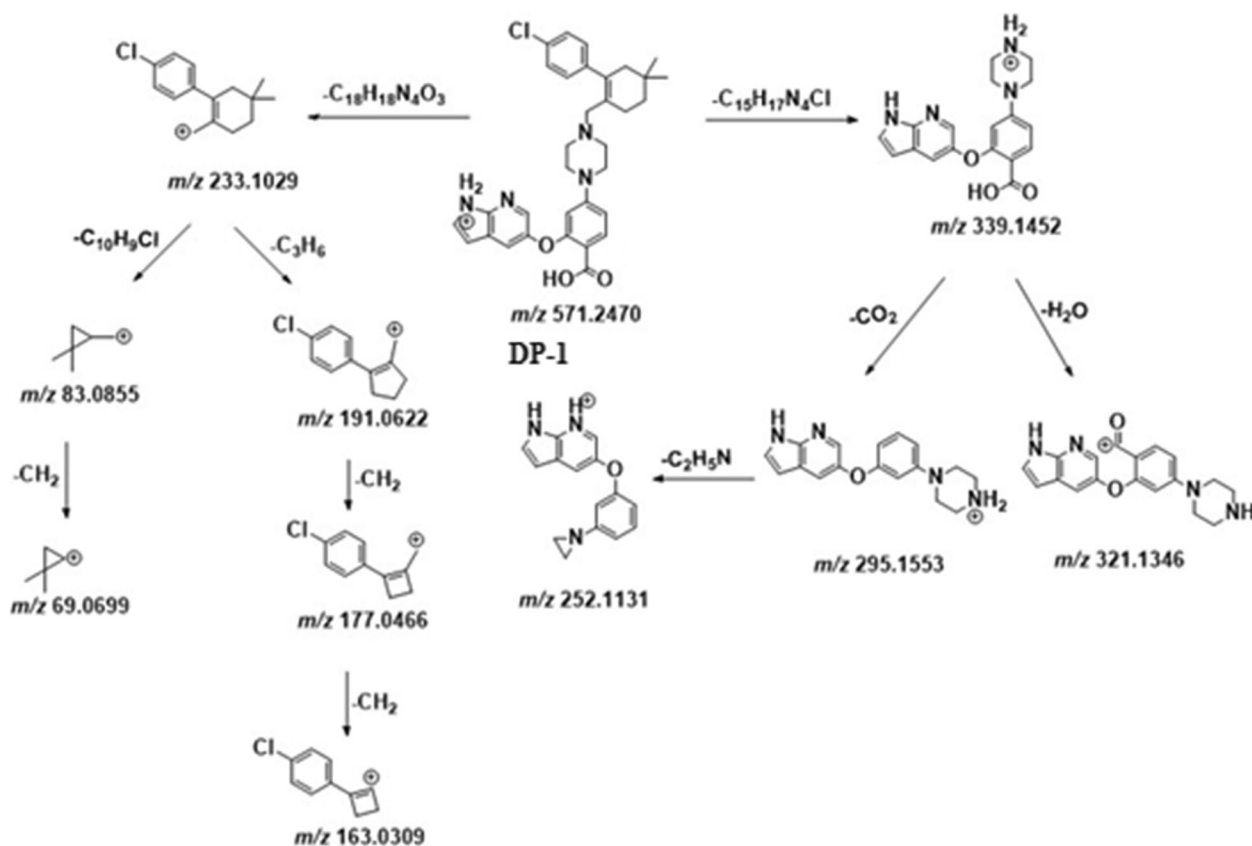
MS/MS spectrum of DP1 formed in acidic hydrolysis (Figure 3b) showed protonated molecular ion at  $m/z$  value of 571.2445 with plausible chemical formula  $C_{33}H_{36}ClN_4O_3^+$ . The fragmentation pattern of DP2 (Scheme 2) revealed major product ions at  $m/z$  339, 321, 233, 177, 163 and 69. Elemental compositions of DP1 and its product ions have been ensured by accurate mass measurements as shown in Table 2. DP1 was identified as 2-((1H-pyrrolo[2,3-b]pyridin-5-yl)oxy)-4-(4-((4'-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-yl)benzoic acid.

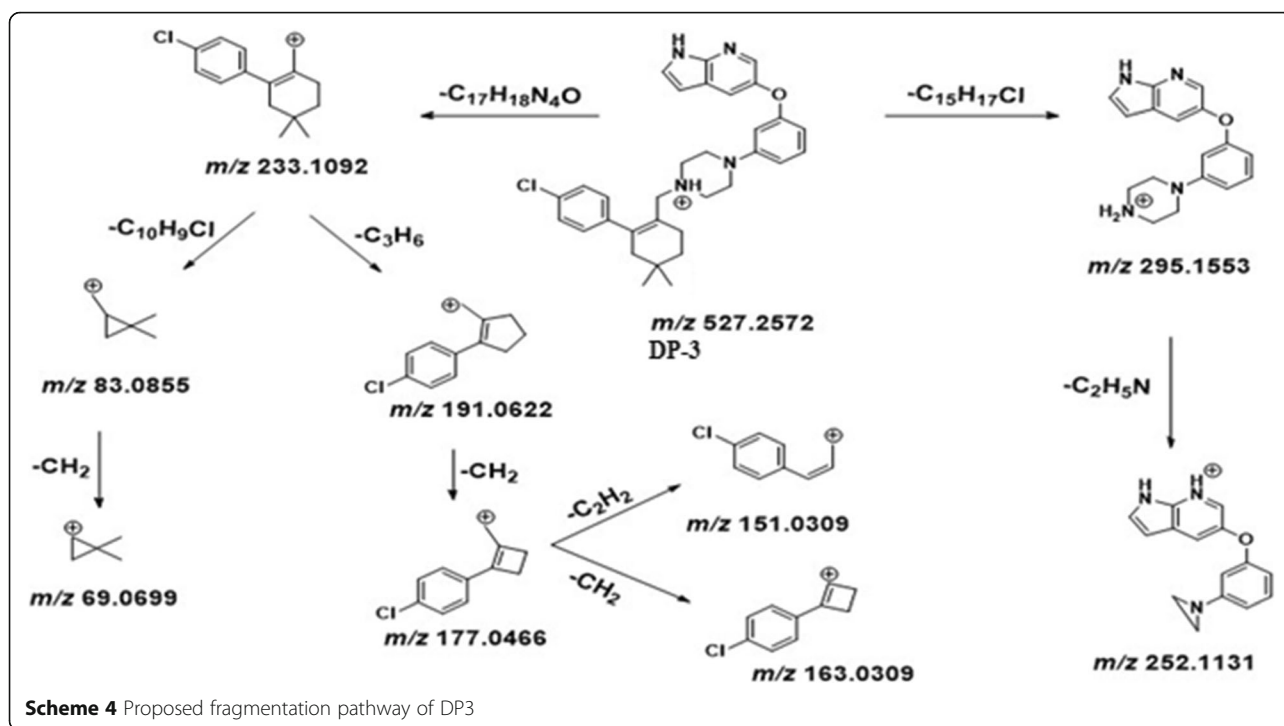


**Table 2** HRMS data of product ions of protonated VEN and its degradation products

	Chemical formula	MS/TOF mass data	Exact mass of most probable structure	ppm error
VEN	C <sub>45</sub> H <sub>51</sub> ClN <sub>7</sub> O <sub>7</sub> S <sup>+</sup>	868.3238	868.3254	-1.84
	C <sub>30</sub> H <sub>34</sub> N <sub>7</sub> O <sub>7</sub> S <sup>+</sup>	636.2268	636.2235	5.18
	C <sub>33</sub> H <sub>34</sub> ClN <sub>4</sub> O <sub>2</sub>	553.2396	553.2365	5.60
	C <sub>18</sub> H <sub>17</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	321.1370	321.1346	7.47
	C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sup>+</sup>	295.1575	295.1553	7.45
	C <sub>15</sub> H <sub>14</sub> N <sub>3</sub> O <sup>+</sup>	252.1144	252.1131	5.15
	C <sub>15</sub> H <sub>18</sub> Cl <sup>+</sup>	233.1111	233.1092	8.15
	C <sub>12</sub> H <sub>12</sub> Cl <sup>+</sup>	191.0633	191.0622	5.75
	C <sub>11</sub> H <sub>10</sub> Cl <sup>+</sup>	177.0480	177.0466	7.90
	C <sub>10</sub> H <sub>8</sub> Cl <sup>+</sup>	163.0321	163.0309	7.36
	C <sub>9</sub> H <sub>8</sub> Cl <sup>+</sup>	151.0321	151.0309	7.94
	C <sub>6</sub> H <sub>11</sub> <sup>+</sup>	83.0866	83.0855	13.23
	C <sub>5</sub> H <sub>9</sub> <sup>+</sup>	69.0707	69.0699	11.58
DP1	C <sub>33</sub> H <sub>36</sub> ClN <sub>4</sub> O <sub>3</sub> <sup>+</sup>	571.2445	571.2470	-4.37
	C <sub>18</sub> H <sub>19</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>	339.1417	339.1452	-10.32
	C <sub>18</sub> H <sub>17</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	321.1315	321.1346	-9.65
	C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sup>+</sup>	295.1533	295.1553	-6.77
	C <sub>15</sub> H <sub>14</sub> N <sub>3</sub> O <sup>+</sup>	252.1144	252.1131	5.15
	C <sub>15</sub> H <sub>18</sub> Cl <sup>+</sup>	233.1069	233.1092	-9.86
	C <sub>12</sub> H <sub>12</sub> Cl <sup>+</sup>	191.0598	191.0622	-12.56
	C <sub>11</sub> H <sub>10</sub> Cl <sup>+</sup>	177.0444	177.0466	-12.42
	C <sub>10</sub> H <sub>8</sub> Cl <sup>+</sup>	163.0290	163.0309	-11.65
	C <sub>6</sub> H <sub>11</sub> <sup>+</sup>	83.0841	83.0855	-16.85
	C <sub>5</sub> H <sub>9</sub> <sup>+</sup>	69.0686	69.0699	-18.82
DP2	C <sub>45</sub> H <sub>51</sub> ClN <sub>7</sub> O <sub>8</sub> S <sup>+</sup>	884.3181	884.3203	-2.48
	C <sub>30</sub> H <sub>34</sub> N <sub>7</sub> O <sub>8</sub> S <sup>+</sup>	652.2156	652.2184	-4.29
	C <sub>18</sub> H <sub>17</sub> N <sub>4</sub> O <sub>3</sub> <sup>+</sup>	337.1278	337.1295	-5.04
	C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sub>2</sub> <sup>+</sup>	311.1487	311.1503	-5.14
	C <sub>15</sub> H <sub>18</sub> Cl <sup>+</sup>	233.1079	233.1092	-5.57
	C <sub>11</sub> H <sub>10</sub> Cl <sup>+</sup>	177.0455	177.0466	-6.21
	C <sub>9</sub> H <sub>8</sub> Cl <sup>+</sup>	151.0315	151.0309	3.97
	C <sub>5</sub> H <sub>9</sub> <sup>+</sup>	69.0692	69.0699	-10.13
DP3	C <sub>32</sub> H <sub>36</sub> ClN <sub>4</sub> O <sup>+</sup>	527.2566	527.2572	-1.13
	C <sub>17</sub> H <sub>19</sub> N <sub>4</sub> O <sup>+</sup>	295.1536	295.1553	-5.75
	C <sub>15</sub> H <sub>14</sub> N <sub>3</sub> O <sup>+</sup>	252.1117	252.1131	-5.55
	C <sub>15</sub> H <sub>18</sub> Cl <sup>+</sup>	233.1076	233.1092	-6.86
	C <sub>12</sub> H <sub>12</sub> Cl <sup>+</sup>	191.0608	191.0622	-7.32
	C <sub>11</sub> H <sub>10</sub> Cl <sup>+</sup>	177.0455	177.0466	-6.21
	C <sub>10</sub> H <sub>8</sub> Cl <sup>+</sup>	163.0298	163.0309	-6.74
	C <sub>9</sub> H <sub>8</sub> Cl <sup>+</sup>	163.0298	151.0309	-7.28
	C <sub>6</sub> H <sub>11</sub> <sup>+</sup>	83.0847	83.0855	-9.62
	C <sub>5</sub> H <sub>9</sub> <sup>+</sup>	69.0692	69.0699	-10.13



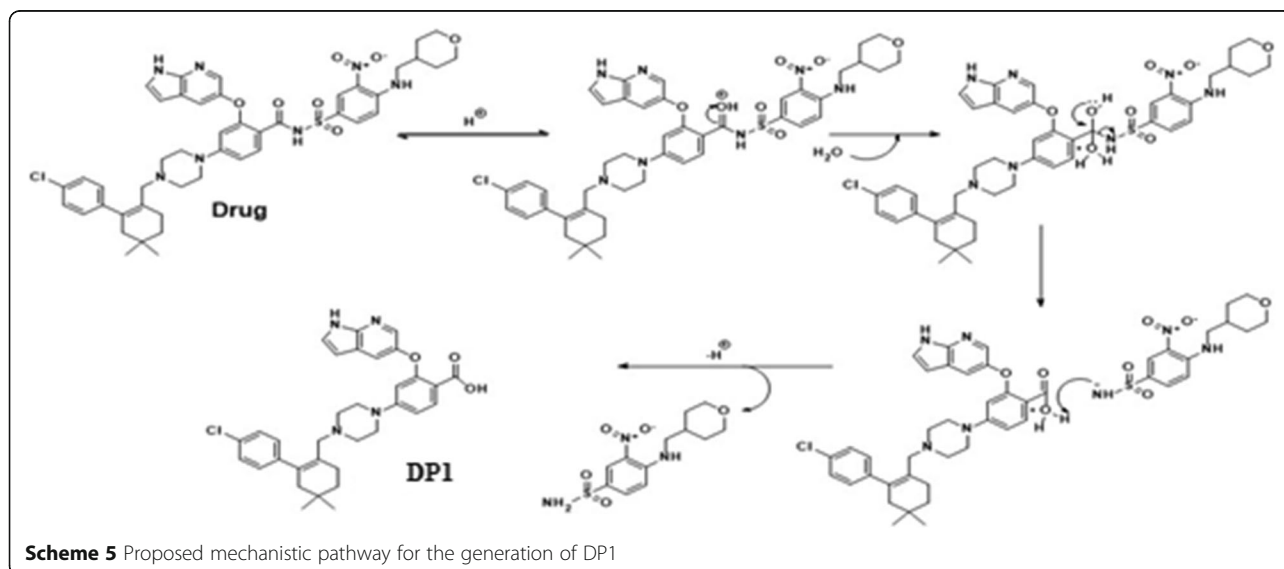


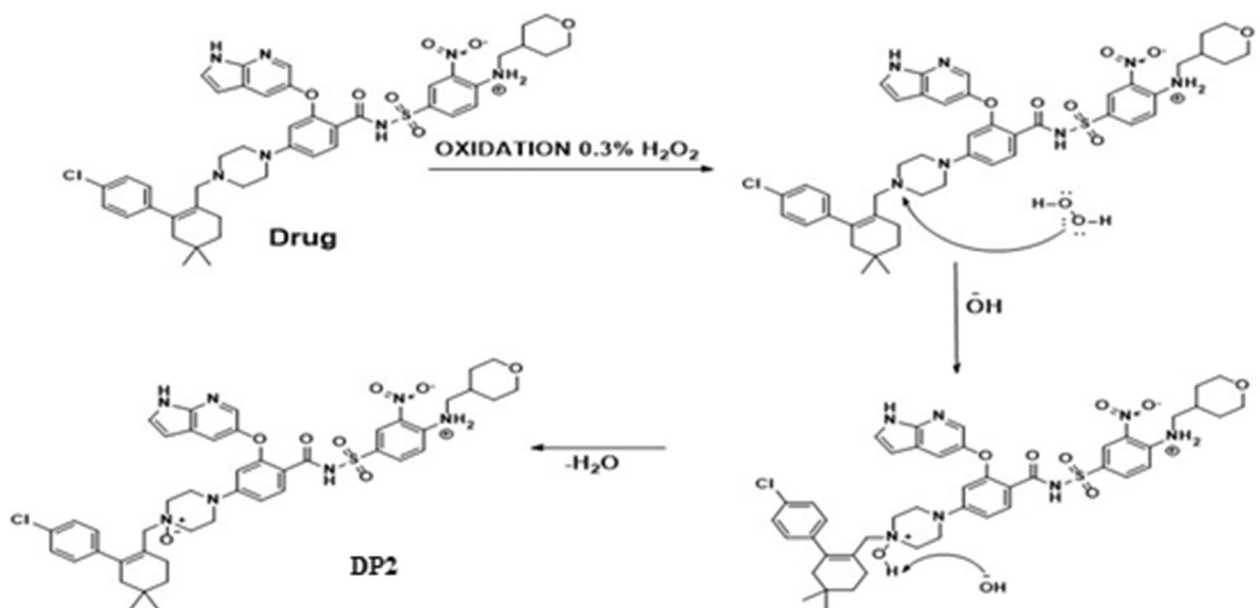


#### MS/MS profiling of DP2

MS/MS spectrum of DP2 formed in oxidative degradation (Figure 3c) showed protonated molecular ion at  $m/z$  value of 884.3181 ( $C_{45}H_{51}ClN_7O_8S^+$ ) in positive ion mode. The mass difference between VEN ( $m/z$  868) and DP2 ( $m/z$  884) was 16 units. We proposed that there was an addition of an oxygen atom to form N-oxide. The fragmentation pattern of DP2 is shown in Scheme 3 with major daughter ions at  $m/z$  652, 337, 233, 177, and 69. Characteristic fragment formed

at  $m/z$  652 (due to chlorophenyldimethyl-cyclohexenyl moiety loss and addition of an oxide ion to nitrogen of piperazine ring) confirms the production of N-oxide. The elemental compositions of DP2 and its product ions have been ensured by accurate mass measurements (Table 2). DP2 was identified as 4-(3-((1H-pyrrolo[2,3-b]pyridin-5-yl)oxy)-4-(((tetrahydro-2H-pyran-4-yl)methyl)amino)phenyl)sulfonylcarbamoylphenyl-1-((4'-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-oxide.





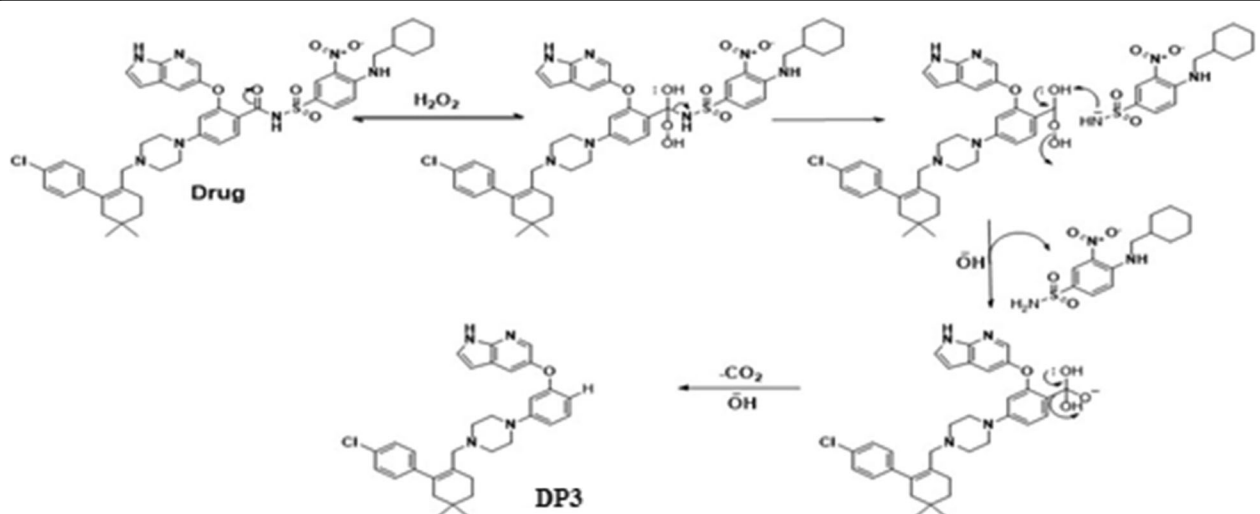
**Scheme 6** Proposed mechanistic pathway for the generation of DP2

#### MS/MS profiling of DP3

MS/MS spectrum of DP3 formed in oxidative degradation (Figure 3d) showed protonated molecular ion at  $m/z$  value of 527.2566 ( $\text{C}_{32}\text{H}_{36}\text{ClN}_4\text{O}^+$ ) in positive ion mode. The fragmentation pattern of DP3 is shown in Scheme 4 with major product ions at  $m/z$  295, 233, 191, 177, 163, 83, and 69. The elemental compositions of DP3 and its product ions was confirmed by accurate mass measurements (Table 2). DP3 was identified as 5-(3-(4-((4'-chloro-5,5-dimethyl-3,4,5,6-tetrahydro-[1,1'-biphenyl]-2-yl)methyl)piperazin-1-yl)phenoxy)-1H-pyrrolo[2,3-b]pyridine.

#### A plausible mechanism of formation of DPs

VEN was degraded into three degradation products in forced degradation experiments. DP1 was formed under acidic hydrolysis by cleavage of an amide bond and further formation of acid. DP2 was formed under oxidative conditions where oxygen was added to nitrogen of a piperazine ring leading to N-oxide formation. DP3 was formed by oxidative decarboxylation under oxidative conditions, where there is a loss of carbon dioxide. The detailed mechanism is shown in Scheme 5, 6 and 7.



**Scheme 7** Proposed mechanistic pathway for the generation of DP3

**Table 3** System suitability study results

System suitability (at 100% level )				
Injections	Area	Asymmetry	Retention time	Theoretical plates
S1	16226464	0.96	22.36	73017
S2	15899107	0.95	22.44	72460
S3	16026113	0.97	22.48	72671
S4	15910984	0.95	22.44	73197
S5	16152639	0.98	22.38	73072
S6	16208396	0.96	22.34	73785
Average	16070617.17	0.96	22.41	73033.67
SD	146203.92	0.01	0.05	459.03
% RSD	0.91%	1.22%	0.24%	0.63%

#### Method validation of VEN

The HPLC method for VEN was validated following ICH Q2 (R2) guideline (ICH 2005). System suitability was determined by injecting a standard solution (n=6) of VEN. Different parameters like retention time, area, asymmetry and theoretical plates were recorded and percent relative standard deviation (RSD) was calculated which was found to be less than 2 (Table 3). The standard linearity plot of peak area (y-axis) versus concentration (x-axis) was plotted by taking seven different concentrations in a range of 50 to 600 µg/mL in triplicate (n=3). Correlation coefficient ( $r^2$ ) was determined through the linear regression equation and was found to be 0.9997. The method accuracy was determined by spiking VEN in an excipient mixture at three different concentration levels of 320, 400, and 480 µg/mL in triplicate (Khan et al. 2020). From the excipient mixture, VEN was extracted, filtered and analyzed. Percentage accuracy was between 99 to 101% at all three concentration levels (Table 4). The method precision was determined by injecting standard concentration for both within (n=6) and between batch (n=12) precision. The %RSD of back-calculated concentration of the standard injections was less than 2 for all the experiments (Tables 5 and 6).

**Table 4** Results of accuracy study

Nominal Concentration (µg/mL)	Average concentration found (n=3) (µg/mL)	SD	RSD (%)	Mean accuracy (%)
320 (80%)	322.69	0.77	0.23	100.84%
400 (100%)	399.72	1.05	0.26	99.93%
480 (120%)	478.78	2.36	0.49	99.74%

**Table 5** Results of within batch precision study

Injections	Concentration (µg/mL)
1	392.00
2	404.40
3	404.92
4	406.25
5	407.66
6	409.12
Average	404.22
SD	5.76
RSD (%)	1.4

#### Conclusion

In this study, a quantitative HPLC assay method has been established for quantification of VEN in presence of their degradation impurities. Stress study of VEN was performed by using different ICH recommended stresses including hydrolysis, oxidation, thermolysis and photolysis. We found three degradation products generated due to acidic hydrolysis and oxidative degradation. DP1 was formed in acidic hydrolysis whereas, DP2 and DP3 were formed in oxidative degradation. VEN was not degraded in basic hydrolysis, neutral hydrolysis, thermal and photolytic stress conditions. DP1, DP2 and DP3 were characterized by LC-Q-TOF-MS and their structures have been proposed based on their accurate mass data. Fragmentation patterns and plausible mechanism of formation of all three DPs have been proposed. This developed stability indicative method can be used in the routine analysis of quality control and stability samples in industry or other research laboratories.

**Table 6** Results of between batch precision study

Injections	Concentration (µg/mL)
1	407.03
2	409.06
3	406.43
4	410.29
5	409.74
6	404.14
7	404.33
8	415.67
9	408.50
10	406.52
11	403.64
12	408.15
Average	407.79
SD	3.31
RSD (%)	0.81

## Abbreviations

VEN: Venetoclax; Bcl-2: B-cell lymphoma 2; USFDA: United States Food and Drug Administration; ICH: International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; HPLC: High performance liquid chromatography; LC-MS: Liquid chromatography-mass spectrometry; Q-TOF: Quadrupole-time of flight; ACN: Acetonitrile; NaOH: Sodium hydroxide; HCl: Hydrochloric acid; DPs: Degradation products; H<sub>2</sub>O<sub>2</sub>: Hydrogen peroxide; UV: Ultraviolet; RSD: Relative standard deviation; r<sup>2</sup>: Correlation coefficient

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## Authors' contributions

Dhruvisha Pokar: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft. Amit Kumar Sahu: Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - review & editing. Pinaki Sengupta: Conceptualization, Investigation, Project administration, Supervision, Visualization, Writing - review & editing. The authors read and approved the final manuscript.

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