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LC–MS/MS characterization of forced degradation products of zofenopril

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ABSTRACT

A rapid, specific and reliable isocratic LC–MS/MS method has been developed and validated for the identification and characterization of stressed degradation products of Zofenopril. Zofenopril, an anti-hypertensive drug, was subjected to hydrolysis (acidic, alkaline and neutral), oxidation, photolysis and thermal stress, as per ICH-specified conditions. The drug showed extensive degradation under oxidative and base hydrolysis stress conditions. However, it was stable to thermal, acid, neutral and photolysis stress conditions. A total of 6 degradation products were observed and the chromatographic separation of the drug and its degradation products were achieved on Phenomenex (Luna) C_{18} (250 mm × 4.6 mm, i.d., 5 μm) column using 20 mM ammonium acetate: acetonitrile (50:50, v/v) as a mobile phase. The degradation products were characterized by LC–MS/MS and its fragmentation pathways were proposed. The LC–MS method was validated with respect to specificity, linearity, accuracy and precision. No previous reports were found in the literature regarding the degradation behavior of zofenopril.

1. Introduction

Zofenopril (ZFN) is an anti-hypertensive drug belonging to the family of the sulphur containing angiotensin-converting enzyme (ACE) inhibitors with cardioprotective properties indicated for the treatment of cardiovascular diseases such as essential hypertension, acute myocardial infarction, heart failure, and slows the development of atherosclerosis [1–4]. Its chemical name is (2S,4R)-1-((S)-3-(benzoylthio)-2-methylpropanoyl)-4-(phenylthio)pyrrolidine-2-carboxylic acid) (Fig. 1). It is a white, crystalline powder. It is a partially polaric nature so freely soluble in water: acetonitrile (50:50, v/v) but partially soluble in polar solvents like water, methanol, etc. ZFN appeared significantly more effective than the older antihypertensive atenolol and enalapril, and was associated with less adverse effects [5,6]. ACE inhibitors have been developed as a further therapeutic action on the renin–angiotensin–aldosterone system, one of the most important regulators of blood pressure [7–9].

Thorough literature search reveals that many HPLC and LC–MS methods for the determination of ZFN in plasma, human serum and other biological matrices were reported. Examples of such analytical methods include enzymatic techniques, radioimmunoassay (RIA) [10], high performance liquid chromatography (HPLC) [11], UV–visible spectrophotometry [12], gas chromatography–mass spectrometry (GC–MS) [13], liquid chromatography–mass spectrometry (LC–MS) and liquid chromatography tandem mass spectrometry (LC–MS/MS) [14–17]. Methods were available for simultaneous determination of ZFN and other antihypertensive in pharmaceutical dosage forms [18–20] and dissolution studies were also reported [21]. Recently, liquid chromatography–mass spectrometry (LC–MS) have evolved as versatile tool for the characterization of drug impurities, degradation products [22,23]. So far, no study has been reported on the systematic characterization and mechanistic pathway of degradation products of ZFN under stress conditions prescribed by ICH Q1A (R2) [24]. The main aim of the present study was to investigate the complete degradation behavior of the drug and to characterize the degradation products. It was accomplished by exposing the drug to ICH-recommended stress conditions of hydrolysis, oxidation, thermal and photolysis, and analyzing the resultant solutions to optimized LC–MS, MS/MS, MS^n and accurate mass measurements to establish the fragmentation pattern of the drug and its degradation products.

2. Experimental

2.1. Chemicals and reagents

Zofenopril (ZFN) (99% purity) was a gift sample from Mylon laboratories Pvt. Ltd. (Hyderabad, India). HPLC grade acetonitrile

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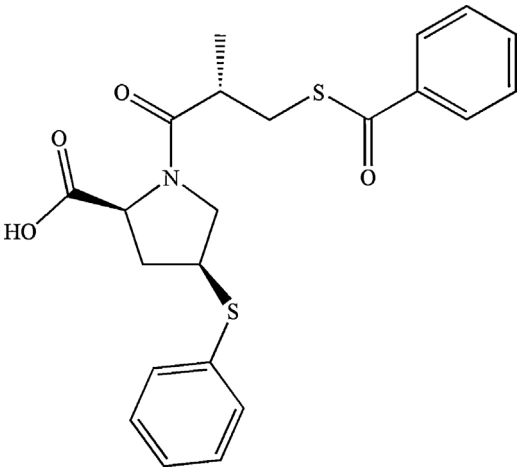

Fig. 1. Chemical structure of ZFN.

was purchased from Rankem (Mumbai, India). Analytical reagent grade sodium hydroxide, hydrochloric acid, hydrogen peroxide and ammonium acetate were purchased from S.D. Fine Chemicals (Mumbai, India). The millipore water used was purified by Millipore synergy (Millipore, France).

2.2. Instrumentation

The HPLC system consisting of two LC-20AD pumps, SPD-M20A diode array detector, SIL-20AC auto sampler, DGU-20A3 degasser, and CBM-20A system controller (all from Shimadzu, Kyoto, Japan) were used. A reverse phase Phenomenex (Luna) C_{18} (250 mm × 4.6 mm, i.d., 5 μm) column was used for separation of all the compounds. The chromatographic data were recorded using an HP-Vectra (Hewlett Packed, Waldron, Germany) computer system with LC solutions data acquiring software (Shimadzu, Kyoto, Japan). LC–MS/MS was performed by Agilent1100 series online ion trap MSD mass spectrometer with APCI source in positive mode equipped with an autosampler (G1329A), and diode array detector (G1315B) (all from Agilent technologies, Waldbronn, Germany). The data were acquired and processed using LC/MSD trap software 4.2 (Bruker, Waldbronn, Germany). The high resolution mass spectrometry (HRMS) data were acquired using a Q-TOF mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, USA), equipped with an ESI source. The data acquisition was under the control of Analyst QS software. MS^n experiments were performed using a quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with an electrospray ionization source. The data acquisition and processing were under the control of Xcalibur software.

2.3. Forced degradation

Stress degradation studies of ZFN were carried out under hydrolysis (acid, base and neutral), oxidation, photolytic and thermal conditions as per ICH guidelines [24]. Acidic and basic hydrolysis was carried out in 1 M HCl, 1 M NaOH, for 24 h and 48 h, respectively, whereas neutral hydrolysis was carried out in water for 72 h. All the hydrolytic studies were conducted at 80 °C with a drug concentration of 1 mg/mL. The oxidative degradation study was carried out with 3% and 6% H_2O_2 at room temperature for 10 days at a concentration of 1 mg/mL. The ZFN substance was spread to about 1 mm thickness in a Petri dish and kept at 100 °C in the oven for 5 days for thermal and exposed to UV light at 320 nm for 10 days for photolytic stress. For thermal stress, the drug was kept at 100 °C in the oven for 5 days. The optimized stressed conditions are outlined in Table 1. All stressed samples were withdrawn at suitable time intervals and diluted up to within the linearity range with mobile phase. All the solutions were filtered using 0.22 μm membrane filters before HPLC and LC–MS analysis.

Table 1
Optimized stress conditions.

Stress condition		Exposure	Duration
Acid	1 M HCl	80 °C	24 h
Base	1 M NaOH	80 °C	48 h
Neutral	H_2O	80 °C	72 h
Oxidation	3% and 6% H_2O_2	Room temperature	10 days
Photolysis	1 mm Petri dish	Ultra-violet light	10 days
Thermal	Oven	100 °C	5 days

2.4. Sample preparation

The degradation products of acid and base hydrolysis were neutralized with sodium hydroxide and hydrochloric acid, respectively. The samples were further diluted up to within the linearity range with mobile phase. Other products of degradation viz., thermal, photolysis, oxidation and neutral hydrolysis were diluted up to within the linearity range with mobile phase. All the samples were kept in refrigerator at 5 °C.

2.5. Mass spectrometric conditions

The mass spectra were recorded in atmospheric pressure chemical ionization (APCI) in positive mode of detection. Nitrogen was the nebulizer and curtain gas. Collision-induced dissociation was achieved by helium as a collision gas. The ion source conditions were set as follows: dry temperature, 325 °C; nebulizer gas, 60 psi; dry gas, 5.0 L/min; capillary exit, 113.5 V; capillary current, 81.787 nA; corona current, 4000 nA; electro multiplier voltage, 2100 V; vaporizer temperature, 400 °C and dwell time, 200 ms. The HRMS data were acquired using a Q-TOF mass spectrometer equipped with an ESI source. The typical source conditions were: capillary voltage, 5.00 kV (positive mode 4 kV); declustering potential, 60 V; focusing potential, 220 V; declustering potential 2, 10 V; resolution 10,000 (full-width half-maximum). Ultra-high pure nitrogen was used as a curtain and collision gas, whereas zero air was used as a nebulizer. For the collision-induced dissociation (CID) experiments, the precursor ion was selected using the quadruple analyzer and TOF analyzer analyzed the product ions.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Initial chromatographic conditions were tried on Waters Symmetry C_{18} column (250 mm × 4.6 mm, i.d., 5 μm) and water:acetonitrile (50:50, v/v) as a mobile phase for the separation of ZFN and its degradation products. The chromatogram showed that the resolution and tailing factor of the degradation products peaks were poor. To improve these parameters, mobile phase of water:acetonitrile with different ratios such as 40:60, 60:40, 65:35, 70:30 and 75:25 (v/v) were tried. These experiments also could not reduce tailing factor. Hence, to get acceptable separation between the drug and its degradation products, ammonium acetate buffer was used instead of water. Aqueous ammonium acetate buffer (20 mM):acetonitrile (50:50%, v/v) in isocratic elution mode and Phenomenex (Luna) C_{18} (250 mm × 4.6 mm, i.d., 5 μm) column were used for successful separation of the ZFN and its degradation products. The flow rate was 0.5 mL/min and detection wavelength was 247 nm. The runtime was 15.0 min. These optimized chromatographic conditions were used for separation

Table 2
Precision and recovery data of ZFN ($n = 5$).

Concentration (ng/mL)	Intra-day precision, measured concentration ± SD; RSD (%)	Inter-day precision, measured concentration ± SD; RSD (%)
20	19.85 ± 0.0394; 0.13	19.86 ± 0.0542; 0.18
40	39.92 ± 0.0345; 0.26	39.90 ± 0.0374; 0.27
60	60.02 ± 0.0354; 0.55	60.11 ± 0.0527; 0.48

Spiked concentration (ng/mL)	Calculated spiked concentration (ng/mL), SD; RSD (%)	Recovery (%)
10	9.96 ± 0.0417; 0.16	99.60
30	30.01 ± 0.0444; 0.34	100.03
50	49.94 ± 0.0397; 0.51	99.88

of ZFN and its degradation products. The method was validated with respect to the parameters outlined in ICH guidelines Q1A (R2). For LC–MS studies, same method was used as for HPLC, without replacement of buffer. The Q-TOF ESI source conditions were also optimized to obtain a good signal and high sensitivity. The conditions like drying gas flow, nebulizing gas flow, drying gas temperature, capillary voltage, spray voltage and skimmer voltage were optimized to maximize the ionization in the source and sensitivity even at a very low concentration to identify and characterize the degradation products.

3.2. Validation

The stability-indicating method was validated for linearity, precision (inter-day and intra-day precision), accuracy and specificity. The optimized LC–MS method was validated with respect to various parameters summarized in the ICH guidelines [24]. Specificity is the ability of the analytical method to measure the analyte concentration accurately in the presence of all the potential impurities. The specificity of the method was established by determining peak purity for ZFN in a mixture of stressed samples using a photodiode array (PDA) detector and evaluation of the resolution factor, and was also demonstrated by subjecting all the degradation samples to LC–MS. The mass detector showed an excellent purity for ZFN and every degradation product, which unambiguously proves the specificity of the method. To establish linearity and range, a stock solution containing 1 mg/mL ZFN in mobile phase was diluted to yield solutions in the concentration range of 10–70 ng/mL. Good linearity was observed in the concentration range 10–70 ng/mL of API. The data were subjected to statistical analysis using a linear regression model; the linear regression equation and correlation coefficient (r^2) were $Y = 13654X + 4590$, 0.9998, respectively. The results have indicated a good linearity. The limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield a signal to noise ratio of 3 for LOD and 10 for LOQ, respectively. The LOD and LOQ values were found to be 4 ng/mL and 13 ng/mL, respectively. The intra- and inter-day precisions were determined at three different concentrations, 20, 40 and 60 ng/mL, on the same day ($n = 5$) and consecutive days ($n = 5$). The procedure was repeated three times over 3 days in order to determine the inter-day accuracy and precision. Table 2 shows that the %RSD for intra and inter-day precision was <15%, indicating that the method was sufficiently precise. Table 2 gives the recoveries of the added drug were obtained from the difference between peak areas of fortified and unfortified degraded samples.

Table 3
Peak purity and chromatographic data of degradation products.

Degradation product	R_t (min)	P.P.I.	R_s	T_f
B_1	2.15	0.9982	6.57	1.12
B_2	2.36	0.9966	5.76	1.58
B_3	2.61	0.9993	4.09	1.17
O_1	1.83	0.9995	6.93	0.98
O_2	4.02	0.9937	7.07	1.23
O_3	6.27	0.9994	7.68	1.57

R_t, retention time; R_s, resolution factor; T_f, tailing factor; P.P.I., peak purity index.

3.3. Degradation behavior

The optimized LC–MS method is applicable for identifying the degradation products. The LC–ESI-MS total ion chromatograms (TIC) obtained under various stress conditions. A total of 6 degradation products were identified and characterized by tandem mass spectrometric analysis (LC–MS/MS). The degradation products are given different notations, viz. B_1, B_2, B_3 and O_1, O_2, O_3 for basic and oxide degradation, respectively (Table 5). Fig. 2 shows the typical chromatograms of the degradation products formed under a variety of stress conditions. The chromatographic parameters, i.e., retention times, resolution, tailing factor and peak purity were determined and given in Table 3.

3.3.1. Hydrolysis

Under base hydrolysis, the drug degraded completely resulting in three degradation products (B_1, B_2 and B_3). However, no degradation was observed under acid and neutral hydrolysis. The degradation products of base hydrolysis were analyzed by LC–MS and the chromatographic data are shown in Table 3.

3.3.2. Oxidation

The drug was oxidized using 3% H_2O_2, and 6% H_2O_2 and for 10 days. It was found that 3% H_2O_2 was ineffective in oxidizing the drug even after 10 days, whereas 6% H_2O_2 could degrade it after 10 days at room temperature. Under these conditions, three degradation products (O_1, O_2 and O_3) were formed.

3.3.3. Photolytic and thermal degradation

The drug was found to be stable in solid as well as in solution forms under UV light and thermal stress. No degradation products were formed.

3.4. MS^n study of zofenopril

The MS^n spectra of ZFN are shown in Fig. 3. At lower collision energy 0.2 mA, protonation of the drug took place and the molecular ion peak at m/z 430 was observed. At high collision energy 0.45 mA, the elimination of S-methyl benzothioate and carbon dioxide from molecular ion peak was observed by the formation of corresponding protonated fragment ions at m/z 386 and 280. The MS^3 studies at collision energy 0.55 mA, revealed that the both ions underwent further fragmentation gave three product ions at m/z 309, 209 and 139 for m/z 386 and two product ions at m/z 204 and 160 for m/z 280. The ions were generated by the loss of neutral molecules viz., benzene, carbon dioxide, C10H13NS and C15H23NOS. The mass spectral fragmentation pathway of ZFN is summarized in Fig. 4. The HRMS data also supported the fragmentation profile of ZFN (Table 4).

3.5. Characterization of degradation products

3.5.1. Base hydrolysis

Zofenopril, on base hydrolysis yielded three degradation products. Table 5 gives the m/z values of the degradants and its

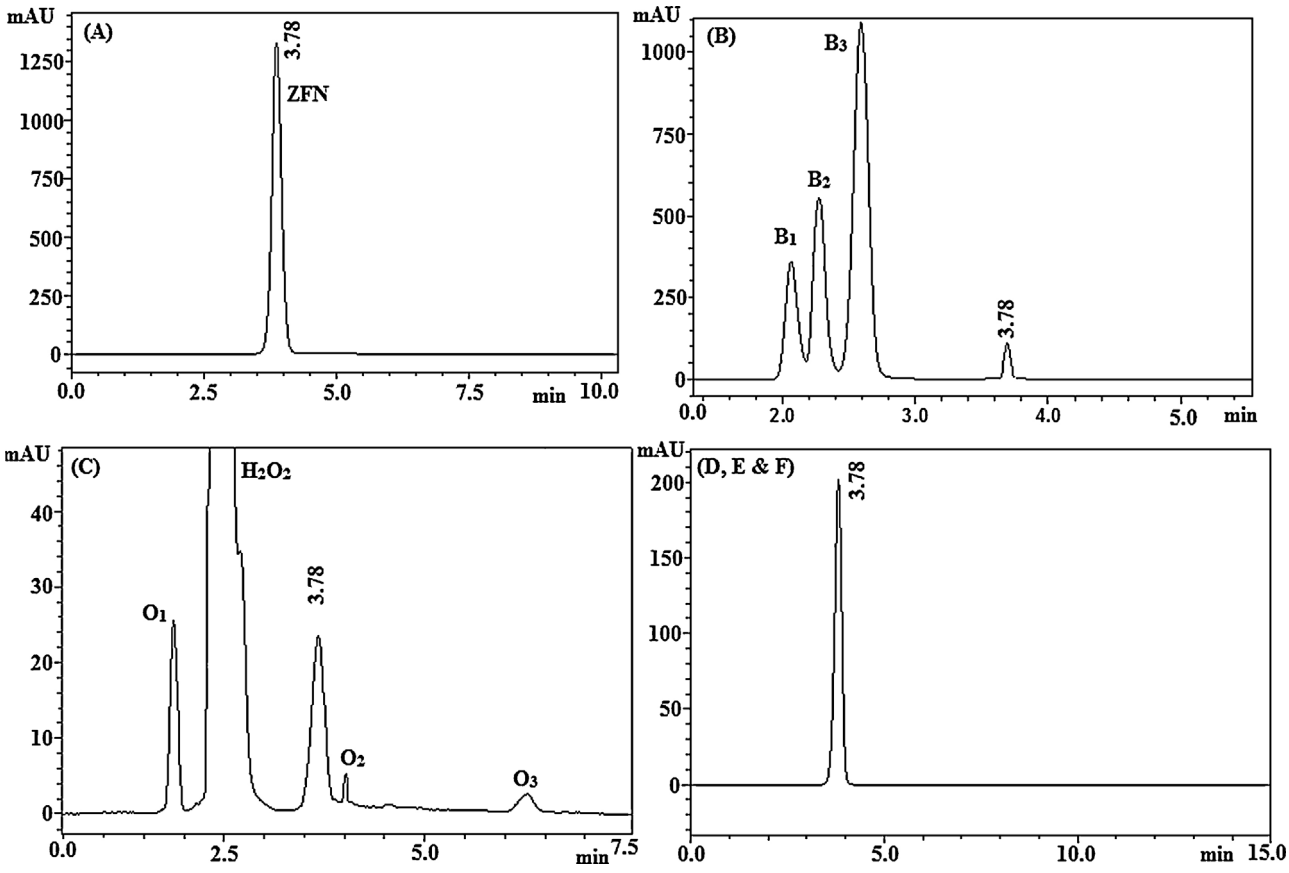

Fig. 2. Typical HPLC chromatograms of (A) standard ZFN and its degradation products under (B) base hydrolysis, (C) 6% hydrogen peroxide, (D) acid hydrolysis, (E)thermal degradation and (F) photolysis.

fragmentation ions. All the basic degradation products of LC–ESI-MS/MS spectra were shown in Fig. 5. The proposed fragmentation pathways for the degradation products of ZFN in basic condition are depicted in Fig. 6. The observed and theoretical accurate mass values for degradation products, along with error in ppm and determined molecular formula of each are given in Table 6. It starts with the reduction of acid group to alcohol from molecular ion resulted to degradant B_2 at m/z 416.0015 Da. The combination of fragment ions m/z 312 and m/z 139 resulted in B_2 (Fig. 6). The same was supported even by their elemental composition, calculated from accurate masses, as $C22H25NO3S2^+$. The molecular ion peak B_1 (326.0741 Da) formed by hydrolysis at benzaldehyde ring leads to the formation of thio alcoholic product. The fragment ion at m/z 312 formed from B_1 by the loss of 16 amu revealed that the precursor ion contains acid group. It followed same fragmentation pattern as the degradation product B_3. In case of B_3, the experimental m/z value was 268.1028 Da and its suggested elemental composition was $C12H13NO4S^+$. Its formation may be best explained by an attack of hydroxide ion on the keto group of ZFN moiety followed by the elimination of m/z 179. The CID MS^2 of m/z 268 ion gave m/z 224

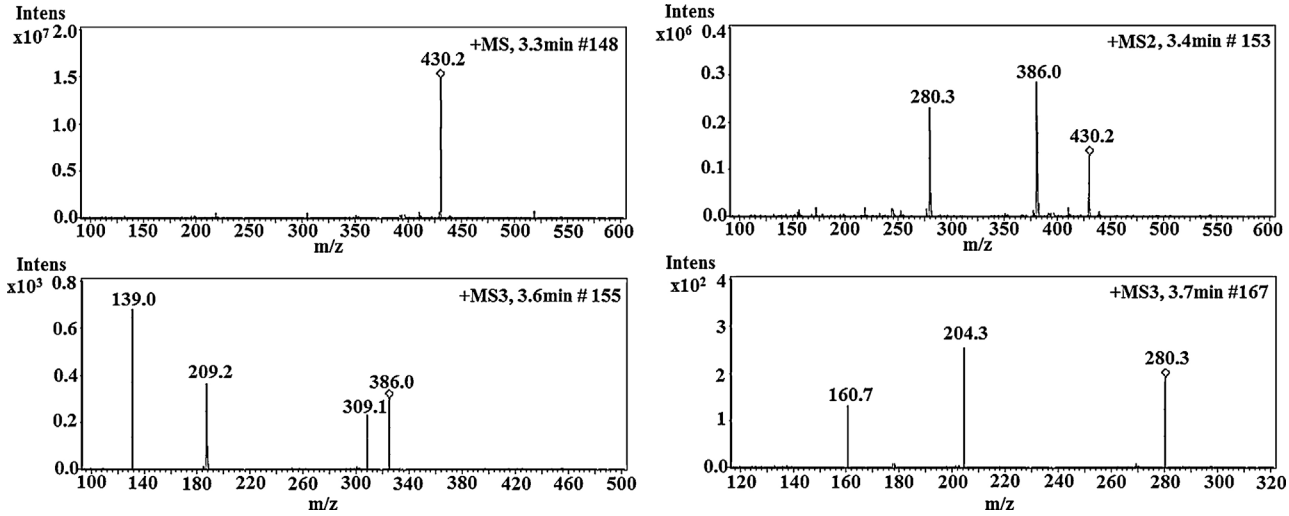

Fig. 3. MS^n fragmentation mass spectra of ZFN ($n = 3$).

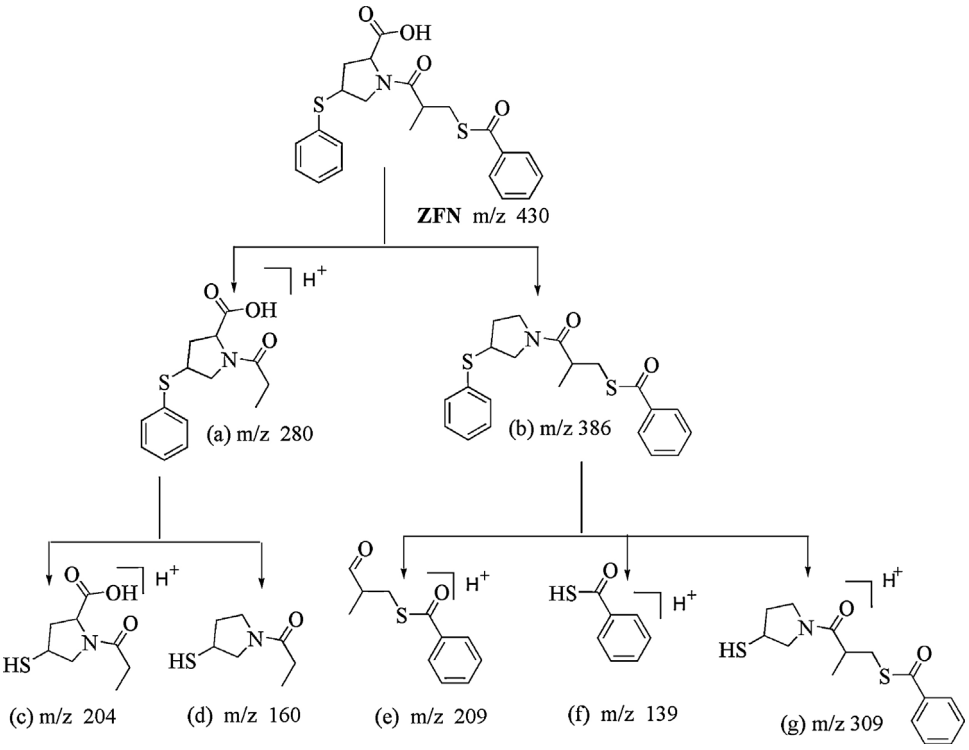

Fig. 4. MS^n fragmentation pathway of ZFN.

(loss of carbon dioxide), and *m/z* 179 (loss of carbon dioxide from *m/z* 224) ions. The accurate mass measurement data and the formation of *m/z* 224 and *m/z* 44 ions may also support the structure of B_1. The peaks B_1, B_2 and B_3 retention times were at 2.1 min, 2.4 min and 2.6 min, respectively, detected by LC–MS. All the above degradation products were supported by the HRMS results as shown in Table 6.

3.5.2. Oxidation

The oxidation of zofenopril yielded three degradation products. All the degradants O_1, O_2 and O_3 were formed with 6% H_2O_2 at room temperature for 10 days. All the oxidation degradation products of LC–ESI-MS/MS spectra were shown in Fig. 5. The oxidation product O_1 has a molecular ion at *m/z*

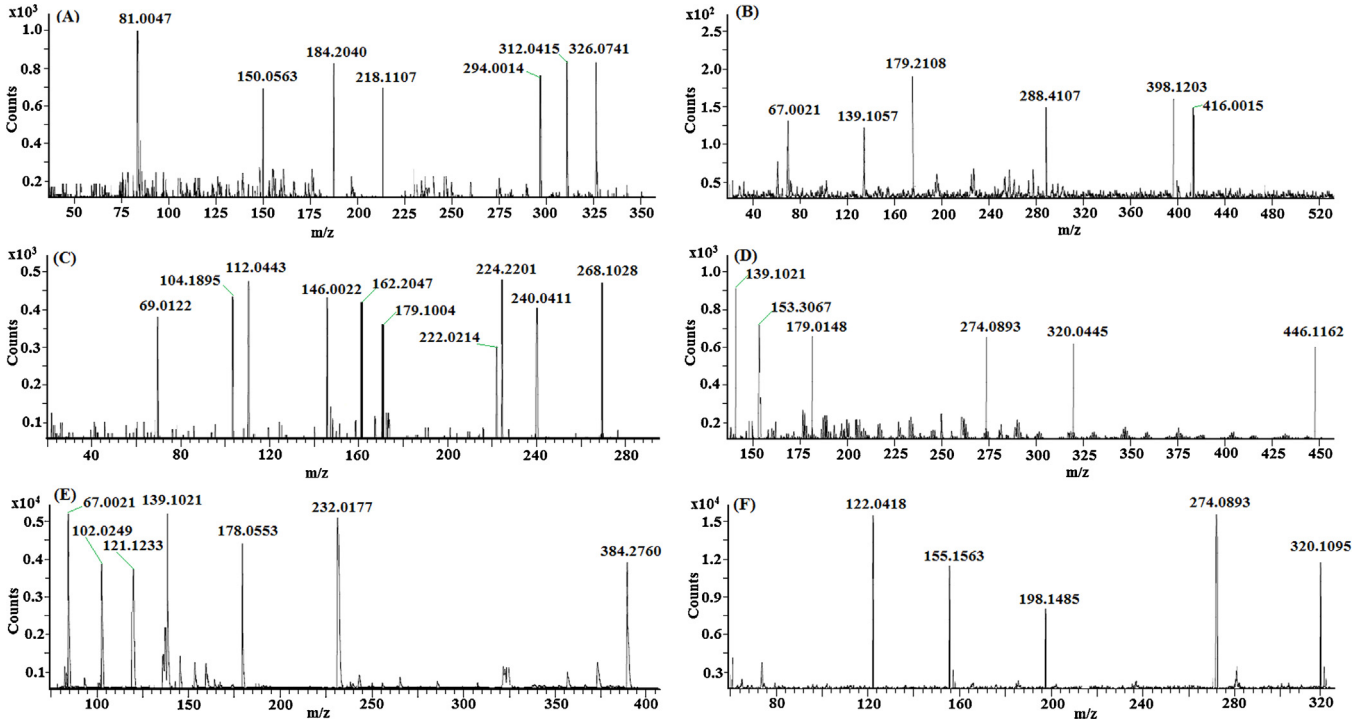

Fig. 5. (A) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 326) of B_1 at 20 eV; (B) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 416) of B_2 at 16 eV; (C) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 268) of B_3 at 25 eV; (D) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 446) of O_1 at 32 eV, (E) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 384) of O_2 at 21 eV; (F) LC–ESI-MS/MS spectrum of $[M+H]^+$ ions (*m/z* 320) of O_3 at 16 eV.

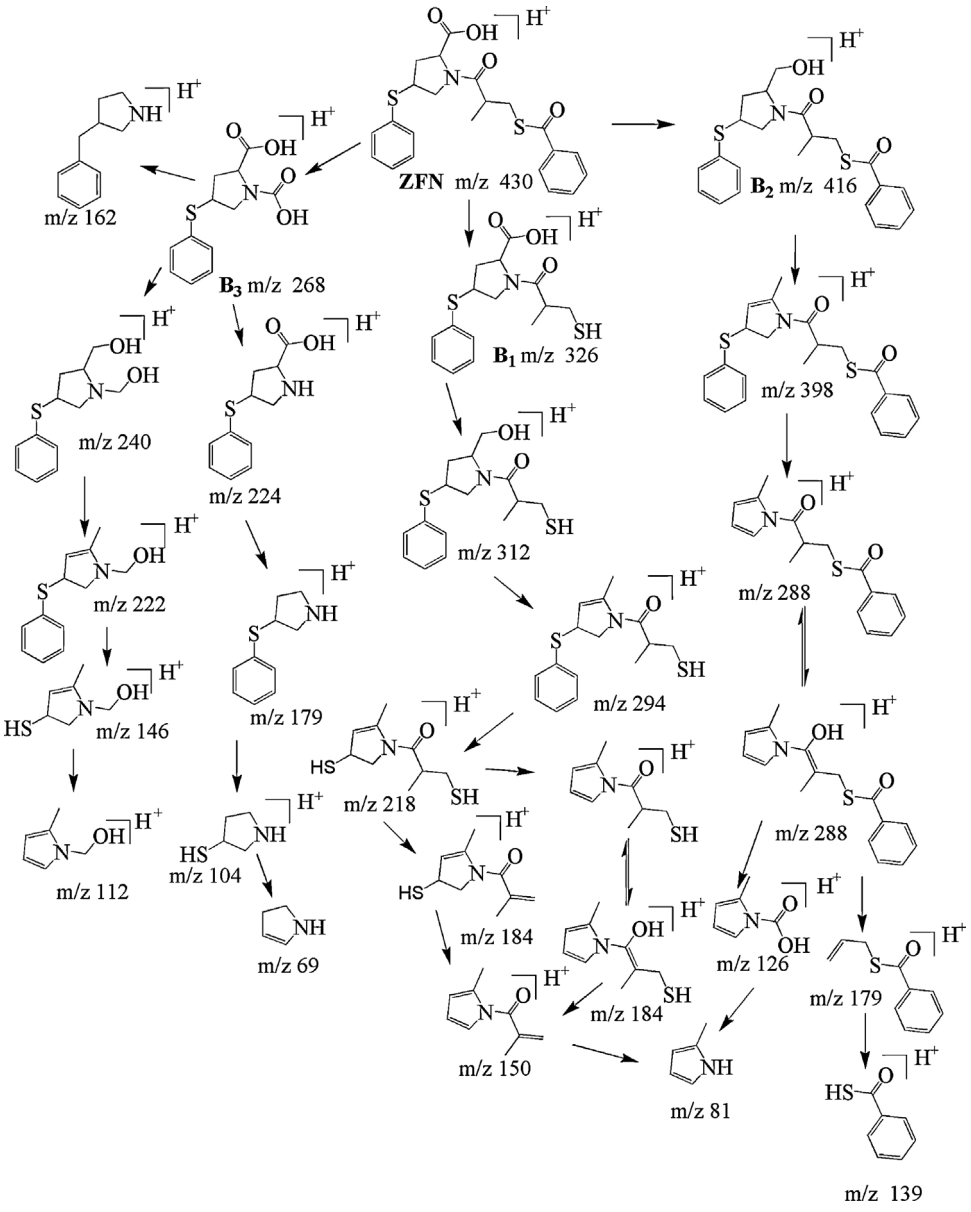

Fig. 6. Proposed fragmentation pathway of degradation products of ZFN under base hydrolysis.

446.1162 Da. Its mass could be attributed due to the formation of N-oxide at pyrrolidine moiety. The MS^2 fragment ions of O_1 were at *m/z* 320, 274, 179, 153 and 139. The elimination of a 2-methyl-1-(1 H-pyrrol-1-yl)propan-1-one molecule from *m/z* 274 yielded *m/z* 139, confirming the presence of thio ester group (Fig. 7). The probable chemical formula given by HRMS is $C22H23NO5S2^+$. The oxidation product O_3 has a molecular ion at *m/z* 320.1095 Da. The degradation product O_3 was formed by the loss of benzenethiol molecules from 3rd position of pyrrolidine moiety. The MS^2 fragment ions of O_3 were at *m/z* 274, 198, 155 and 122. The second oxidation product O_2 was observed at *m/z* 384.2760. It was formed simply by the loss of a formic acid group. The further MS/MS fragments at *m/z* 232, 178, 139, 121, 102, and 67 and also, the HRMS results (Table 6) supported the

Table 4
HRMS data of ZFN and its fragment ions.

Fragmentation	Observed ion mass *m/z* (amu)	Best possible molecular formula	Theoretical mass *m/z* (amu)	Error (ppm)
$[M+H]^+$	430.1576	$C22H23NO4S2^+$	430.0845	−0.016
a	280.0784	$C14H17NO3S^+$	280.1020	0.842
b	386.1143	$C21H23NO2S2^+$	386.1107	−9.323
c	204.0912	$C8H13NO3S^+$	204.0521	−0.019
d	160.0651	$C7H13NOS^+$	160.0105	−0.034
e	209.1184	$C11H12O2S^+$	209.1089	−0.004
f	139.0037	$C7H6OS^+$	139.0172	0.971
g	309.0374	$C15H19NO2S2^+$	309.0546	0.556

Table 5
m/z values of degradants and its fragment ions of ZFN.

Degradation product	Molecular ion *m/z* (amu)	Fragment ions *m/z* (amu)
B_1	326	312, 294, 218, 184, 150, 81
B_2	416	398, 288, 179, 139, 67
B_3	268	240, 224, 222, 179, 162, 146, 112, 104, 69
O_1	446	320, 274, 179, 153, 139
O_2	384	232, 178, 139, 121, 102, 67
O_3	320	274, 198, 155, 122

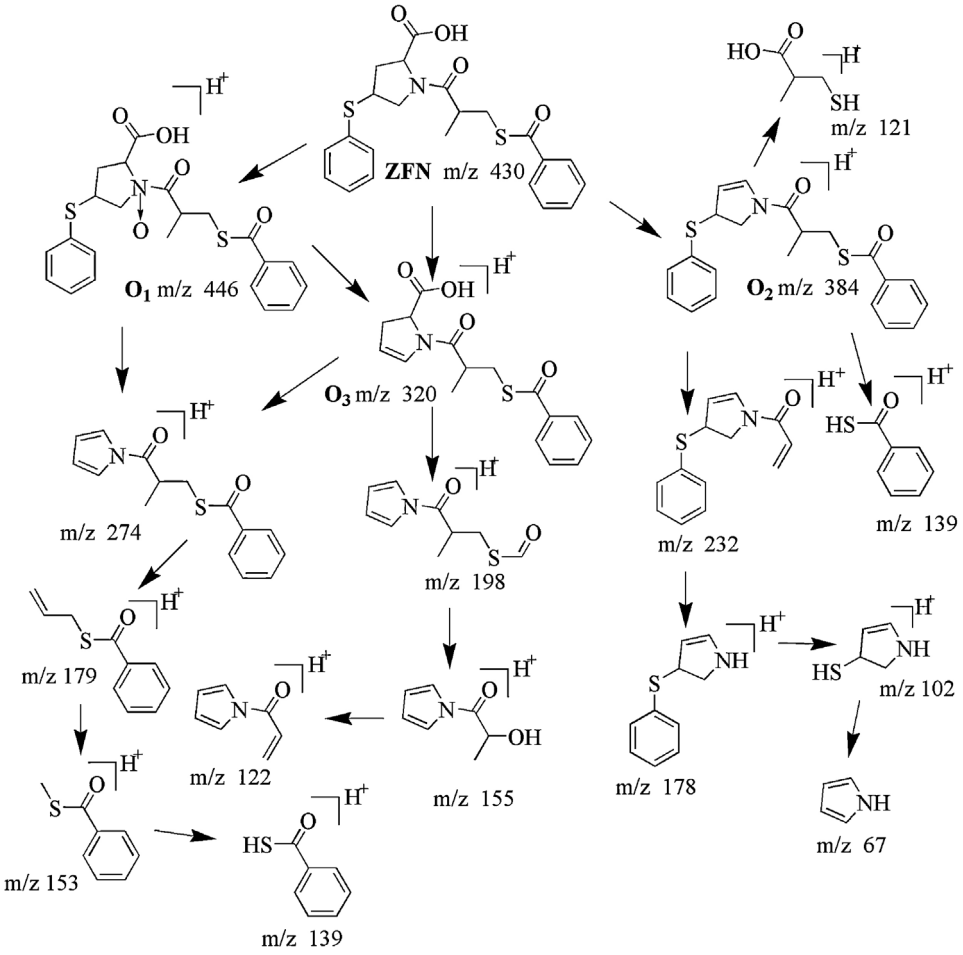

Fig. 7. Proposed fragmentation pathway of degradation products of ZFN under oxidation.

Table 6
HRMS data of degradation products of ZFN.

Degradation product	Theoretical mass *m/z* (amu)	Most probable molecular formula	Observed mass *m/z* (amu)	Error (ppm)
B_1	326.0741	$C15H19NO3S2^+$	326.0736	−1.533
B_2	416.0015	$C22H25NO3S2^+$	416.0022	0.016
B_3	268.1028	$C12H13NO4S^+$	268.0994	−0.001
O_1	446.1162	$C22H23NO5S2^+$	446.1172	0.024
O_2	384.2760	$C21H21NO2S2^+$	384.2684	−0.001
O_3	320.1095	$C16H17NO4S^+$	320.0984	−0.003

proposed structure. All the oxidative degradation products and their fragmentation pattern are shown in Fig. 7.

4. Conclusions

A validated LC–MS/MS method for stability indicating assay of ZFN was developed. The degradation behavior of Zofenopril under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions was carried out according to ICH guidelines. The liquid chromatography method described in the present study can resolve all the degradation products from the ZFN as well as from each other under various stress conditions. The drug showed extensive degradation in base hydrolysis and oxidative stress, while it was stable to acid, neutral, thermal and photolytic stress conditions. A total of 6 degradation products were characterized and the fragmentation pathways were proposed based on LC–MS/MS data and HRMS results.

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