ISSN: 2357-0547 (Print)
ISSN: 2357-0539 (Online)

Research Article / JAPR
El-Leithy et al., 2017, 1 (4), 201-208

Journal of Advanced Pharmacy Research

Characterization of Degradation Products Resulted from Acidic Hydrolysis of Lisinopril Under Drastic Conditions

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Submitted on: 13-07-2017; Revised on: 02-09-2017; Accepted on: 15-09-2017

ABSTRACT

Objective: Characterization and structure elucidation of the degradation products which resulted from the acidic hydrolysis of lisinopril under drastic condition. **Method:** Ultra-performance liquid chromatography coupled with mass/mass spectroscopy (LC-MS/MS) with simple and sensitive method was used to detect the formed degradation products. The fragmentation patterns of the degradation products were investigated. **Results:** The validation of analytical method was satisfied to the recommend criteria of international conference of harmonization. Four degradation products were formed due to the acidic hydrolysis of lisinopril. **Conclusion:** Two degradation products were described in E. Ph. (8th) as impurities. While both of 6-amino-2-((1-carboxy-3-phenylpropyl)amino) hexanoic acid (m/z 309^+) and 1-(2,6-diamino-hexanoyl)pyrrolidine-2-carboxylic acid (m/z 244^+) were formed and identified.

Keywords: *Chemical structure elucidation; Degradation products; LC-MS/MS; Lisinopril analysis.*

INTRODUCTION

Lisinopril,2*S*)-1-[(2*S*)-6-amino-2-{[(1*S*)-1-carboxy-3-phenylpropyl] amino}hexanoyl] pyrrolidine-2 carbo-xylic acid is an angiotensin converting enzyme (ACE) inhibitor, used for the treatment of hypertension, heart failure, and acute myocardial infarction[1-2]. A wide variety of separation and detection techniques have been applied to the analysis of lisinopril, such as gas chromatography coupled with mass spectrometry (GC/MS)[3-4], high performance liquid chromatography (HPLC)[5-6], and high performance liquid chromatography coupled with mass spectrometry (HPLC/MS)[7-8]. The degradation profile of a drug substance is critical to its safety assessment and formulation process in addition to dosage form administration. For safety reasons, the degradation products or impurities of a drug that exceed 0.1% must be identified prior to clinical trials[9]. This paper presents a modified validated method for determination of lisinopril with high resolving from the formed degradation products or impurities by LC-MS/MS in addition to identification of the four formed degradation products by employing LC-MS/MS analysis technique and studying the fragmentation behavior of the resulted product ions to elucidate their chemical structures.

MATERIALS AND METHODS

Materials

Lisinopril certified reference standard (CRS), USA Pharmacopeia, USA. Lisinopril raw material was purchased from Sigma aldrish, Germany. Formic acid was from Sharlu, Spain; Acetonitrile and methanol HPLC grade were brought from J. T. Baker, USA. All other reagents were of analytical grade. Distilled

water was obtained from jancon distillation equipment and filtered using 0.2 µm millipore filtration system.

Instrumentation

Digital balance, Keren, Germany; Magnetic stirrer, Thermo, USA; Ultra-sonicator path, Jenway, UK; UPLC chromatographic C18 column (1.7µm 2.1X 100 mm) end capped Ethylene Bridged Hybrid (BEH), Waters, USA; LC-MS/MS model UPLC Acquity auto sampler, binary solvent pump, Tandem TQ mass detector, and Mass lynx V4.1softwar integrator, Waters, USA.

Mass spectroscopy conditions

LC-MS/MS separation was performed on 40° C with injection volume of 2µl on C18 (1.7µm 2.1X 100 mm) end capped (BEH). Mobile phase flow rate was 0.2 ml/min. The optimum TQ mass spectrometer parameters were equipped as following: the ionization process was done by ESI in positive ion mode, Source temperature was at 120°C, drying nitrogen was used as dissolving gas at flow rate of 700 l/h and dissolving temperature was at 400°C, Capillary voltage was at 3Kv and cone voltage at 40 Kv, dwell time per transition was 0.112 (sec), and ion transition for screening degradation products fragments was at m/z 1000. The selected collision energy value for every degradation product was applied to screen its fragment ions pattern with accepted relative abundance values for molecular characterization.

Method

Preparation of mobile phase

The mobile phase was prepared according to Zhou *et al.*,[10]with a slight modification in its composition (aqueous to organic ratio) to resolve lisinopril and degradation products. It consists of an acidified aqueous solution with formic acid (pH 2.9): methanol: acetonitrile in a ratio of (75: 15: 10, v/v), respectively. The mixture was stirred continuously for 30.0 min to ensure complete equilibrium. The final mobile phase solution was filtered on 0.2µm millipore filtration system and degassed on ultra-sonicator path for 20.0 min to get rid of air bubbles.

Preparation of serial standard solutions

A stock solution was prepared by dissolving a respective weight of Lisinopril CRS in mobile phases to obtain a concentration of 5µg/ml. Serial concentrations of 50, 100, 150, 200, 250,300 and 350 ng/ml were prepared from the stock solution to construct a calibration curve.

Preparation of sample solutions

Serial concentrations of sample solutions were skipped with 50 ng/ml of lisinopril standard solution to obtain concentrations of 150, 200 and 250 ng/ml.

Acidic hydrolysis of lisinopril

Lisinopril solutions of 50 µg/ml concentration were exposed to acidic hydrolytic degradation under the effect of 0.1 N HCl for 4 h at a temperature of 90°C. At the end of the experimental period, the solutions were diluted with the mobile phase and filtered through 0.2µm membrane to be injected into LC-MS/MS.

Validation of the analytical method

Validation of the analytical method was performed according to the international conference of harmonization guidelines (ICH) [11] for terms of linearity, accuracy, range, precision, specificity, sensitivity, and robustness and governed by United States pharmacopeia [USP 38 NF 29][12] criteria for chromatographic separation.

Identification of the degradation products using LC-MS/MS

LC-MS/MS was employed for characterization of the degradation products[13] resulting from exposure of lisinopril to drastic hydrolysis conditions. First; the study was based on injecting lisinopril standard solution at concentration 200 ng/ml into LC-MS/MS system for determining the relative standard deviation percentage %RSD to confirm the precision and integrity of LC-MS/MS system conditions. Second; the study was extended to determine and follow the fragmentation pattern pathway of the degradation products which formed under the effect of acidic hydrolysis conditions of 0.1N HCl at temperature 90 °C for 4 h on lisinopril molecule. Three replicates were obtained from the acidic hydrolysis of lisinopril and diluted with the mobile phase, filtered through 0.2 µm membrane filter and injected in duplicates into the LC-MS/MS system.

Ion transition of screen spectrum was applied to m/z 1000 to detect the main separated degradation products and subsequently followed by running the spectrum to m/z 600 for every degradation product. The collision energy in a range of 10-35v at a fixed cone value was screened for every degradation product to select the appropriate value, which enables the detection of the fragment ions with accepted relative abundance value for degradation products characterization.

RESULTS AND DISCUSSION

Validation of the analytical method

Table 1 summarized the results of the validation of the analytical method of lisinopril analysis

by LC-MS/MS which revealed a high linear calibration curve as represented by **Figure 1** at a range between 50-350 ng/mL with mean recovery 99.93% of SD ± 0.42 for the spiked samples. The precision of the method evaluated by means of the relative standard deviation percentage (%RSD) of intraday ruggedness between two analysts at three different times per day (morning, noon, evening) was 0.29 and % RSD inter-day ruggedness between two analysts at three successive days was 0.74. The sensitivity of the method was represented by limit of detection 9.38 ng/ml and limit of quantitation 28.43ng/ml. The method is robust and can tolerate different labs variations according to robustness studies results. Specificity of the method to separate lisinopril from its degradation products was achieved with resolving of lisinopril from its degradation products with resolution factor of 3.28. System suitability of the validated method was accepted as described in **Table 2**.

Table 1. Validation of the analytical method results for lisinopril determination by LC-MS/MS.

Validation of the analytical method parameters		Results
Linearity	Correlation coefficient R^2	0.9990
	Slope	1530.5
	Intercept	10013
Accuracy	Mean % recovery	99.93 % ± SD 0.32
Range		50:350 ng/ml
Precision	Intraday (RSD %)	0.19
	Interday (RSD %)	0.74
Sensitivity	Limit of detection	14.91ng/ml
	Limit of quantitation	45.18ng/ml
Specificity	Recovery % in 0.1 N HCl	82.56 % ± SD 0.68
	Recovery % in 0.1 N NaOH	97.28 %± SD 0.54
	Recovery % in 10% H_2O_2	93.86 % ± SD 0.96
Robustness	pH 2.4 (RSD%)	0.37
	pH 3.4 (RSD%)	0.45
	Flow rate 0.14 mL/min (RSD %)	0.21
	Flow rate 0.24 mL/min (RSD %)	0.26
	Column temperature 45 (RSD %)	0.22

Table 2. system suitability results of the chromatographic method for lisinopril separation and analysis by LC-MS/MS.

System suitability parameter	Result	USP 38 NF 33 Criteria
Number of theoretical plates	6050	Not less than 2000
Tailing factor	1.24	Not more than 2
Capacity factor	3.57	Not Less than 2

Studying the fragmentation pattern of lisinopril and its degradation products

LC-MS/MS could identify the degradation products of lisinopril by studying its fragmentation patterns represented by the mass spectrum [14-15]. First, protonated lisinopril [M+1] (m/z 406^+) fragments under collision induced by dissociation energy (CID) of 27.21v showed maximum relative abundance ion products at (m/z 246^+) and (m/z 84^+) as shown in **Table 3** and **Figure 3**. The spectrum of protonated lisinopril (m/z 406^+) shows a product ion at [$406^+ \rightarrow 360^+$] resulting from elimination of formic acid in a single step with a larger possibility than consecutive loss of water then carbon monoxide as there was no signal detected for water loss [$406^+ \rightarrow 388^+$]. The signal of (m/z 309^+) resulted from elimination of dihydropyrrole and carbon monoxide and migration of hydroxyl function group[16,17], it can't be obtained the elimination process produced two molecules of dihydropyrrole and carbon monoxide or one neutral compound of dihydropyrrole carbaldehyde as there was no signals for their precursors.

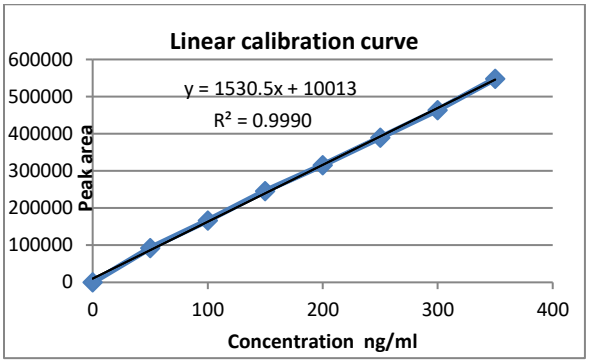

Figure 1. Linear calibration curve of lisinopril concentrations and their corresponding peak areas.

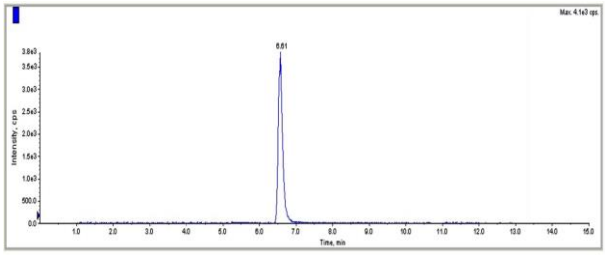

Figure 2. Separation of lisinopril (200ng/ml) after applying modified chromatographic conditions for LC-MS/MS analysis at m/z 406^+ in positive ion mode; it shows retention time of lisinopril at 6.61.

Signal of (m/z 291^+) is resulted from cleavage of dipeptide bond and formation of N-terminal b [$H_2NCH(R)CO$][18] because of elimination of dihydropyrrole with carboxylic terminal and formation

of seven membrane ring and cyclization of the N-terminal.

The signal at (m/z 263$^+$) resulted from the elimination of carbon monoxide and Pyrrolidine-2-carboxylic acid moieties from the product ion [406$^+$]. The product ion of [m/z 246$^+$] resulted from elimination of the amino group from the [m/z 263$^+$]. The signal of the maximum relative abundance at (m/z 84$^+$) indicates the formation of tetrahydropyridine ring after cyclization of the butyl side chain of the lysine residue with the imine group and its release from the product ion of [m/z 246$^+$][17] as represented in **Figure 4**.

Table 3. Summary of chemical formulae, relative abundance, measured and exact mass of major ions observed in the product ion MS/MS spectrum of [M+ H] (m/z 406).

Chemical formulae	Relative Abundance	Measured mass m/z [H+1]	Exact mass m/z [H+1]	Difference between measured and exact mass
$C_{21}H_{31}N_3O_5$	41.57 ±SD 0.13	406.362	406.230	0.132
$C_{20}H_{29}N_3O_3$	8.43 ±SD 0.27	360.325	360.228	0.097
$C_{16}H_{24}N_2O_4$	32.13 ±SD 0.18	309.381	309.174	0.207
$C_{16}H_{22}N_2O_3$	6.83 ±SD 0.3	291.372	291.166	0.206
$C_{15}H_{23}N_2O_2^+$	19.68 ±SD 0.22	263.91	263.175	0.735
$C_{15}H_{20}NO_2^+$	54.22 ±SD 0.14	246.561	246.148	0.413
$C_5H_{10}N^+$	79.52 ±SD 0.09	84.259	84.081	0.178

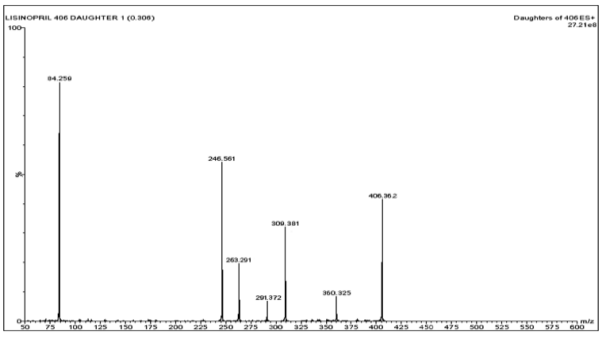

Figure 3. Product ion collusion induced dissociation (CID) mass spectrum for [M+1] ion (m/z 406$^+$) of lisinopril.

The formed degradation products as a result of acidic hydrolysis of lisinopril had been separated and detected by LC-MS/MS as shown in **Figures 4** and **5** respectively. It was observed that the product ions of lisinopril as well as each formed degradation products appeared at mass peaks of m/z [M+1] of 406$^+$, 388$^+$, 309$^+$, 244$^+$ and 180$^+$.

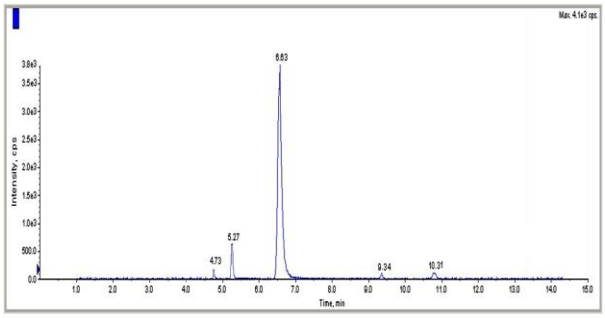

Figure 4. Separation of lisinopril and the resulted degradation products showed the main peak of lisinopril at 6.63 min and the peaks of degradation products at 4.79, 6.27, 9.34 and 10.31 min.

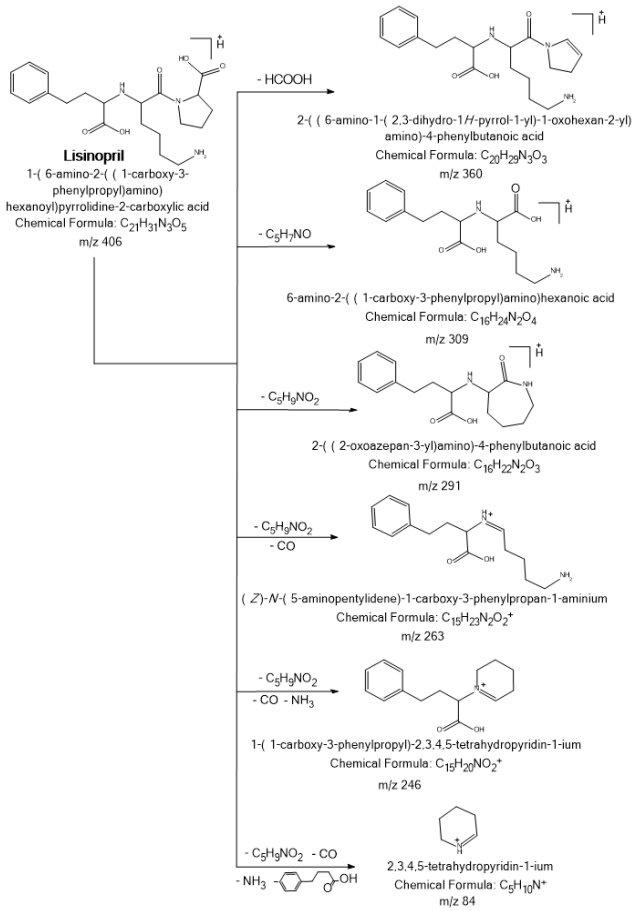

Figure 4. Possible schematic mechanism of the fragmentation pattern pathway of the product ion (m/z 406$^+$) from lisinopril.

Figure 7 shows the fragmentation pattern of the protonated degradation product (m/z 388$^+$) which has been investigated in **Figure 8** and revealed that the product ion (m/z 388$^+$) resulted from the elimination of a water molecule from lisinopril and formation of

piperazine ring with two carbonyl groups. The fragmentation pattern of the protonated product (m/z 388^+) under CID energy of 24.5v shows peaks at (m/z 343^+) due to the elimination of formic acid, (m/z 208^+) due to the elimination of the but-3-en-1-ylbenzene group with the amino group of the lysine residue and cyclization of the lysine residue to form the tetrahydropyridine ring attached iopiperazin ring, (m/z 182^+) due to elimination of a carbonyl group, and (m/z 84^+) due to elimination of both pyrrolidine and carbonyl group to give tetrahydropyridine moiety as presented in **Table 4**.

Table 4. Summary of chemical formulae, relative abundance, measured and exact mass of the major ions observed in the product ion MS/MS spectrum of [M+ H] (m/z 388^+).

Chemical formulae	Relative Abundance	Measured mass m/z [H+1]	Exact mass m/z [H+1]	Difference
$C_{21}H_{29}N_3O_3$	30.83 ±SD 0.11	388.421	388.21	0.211
$C_{20}H2_8N_3O_2^+$	16.51 ±SD 0.24	343.436	343.21	0.226
$C_{11}H_{15}N_2O_2^+$	62.25 ±SD 0.36	208.321	208.11	0.211
$C_{10}H_{17}N_2O^+$	21.29 ±SD 0.18	182.481	182.13	0.351
$C_5H_{10}N^+$	90.67 ±SD 0.21	84.581	84.08	0.501

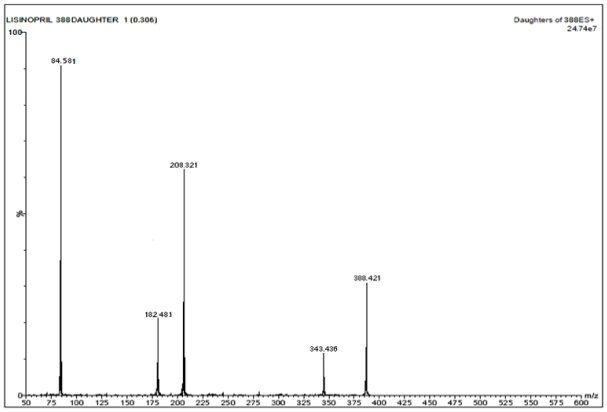

Figure 7. MS/MS spectrum of the product ion [M+1] $C_{21}H_{29}N_3O_3$ at m/z (388^+).

MS/MS spectrum of the product ion (m/z 309^+) was represented by **Figure 9** and studied in **Figure 10**. The protonated degradation product (m/z 309^+) resulted from hydrolysis of lisinopril and removal of pyrrolidine ring and carbon monoxide with migration of hydroxyl group to form with carbonyl group a carboxylic group[16,17].

The fragmentation pattern of (m/z 309^+) under CID energy of 28.4v shows peaks at (m/z 246^+) due to elimination of formic acid with the amino group and cyclization of the lysine residue to from a tetrahydropyridine ring, (m/z 200^+) due to elimination of formic acid, (m/z 142^+) is the same pathway for formation of the product ion of (m/z 246^+) with elimination of the styrene[19], and (m/z 84^+) due to elimination of CH_2COOH group from product ion of (m/z 142^+) or elimination of the but-3-en-1-ylbenzene group from the product ion of (m/z 201^+) as presented in **Table 5**.

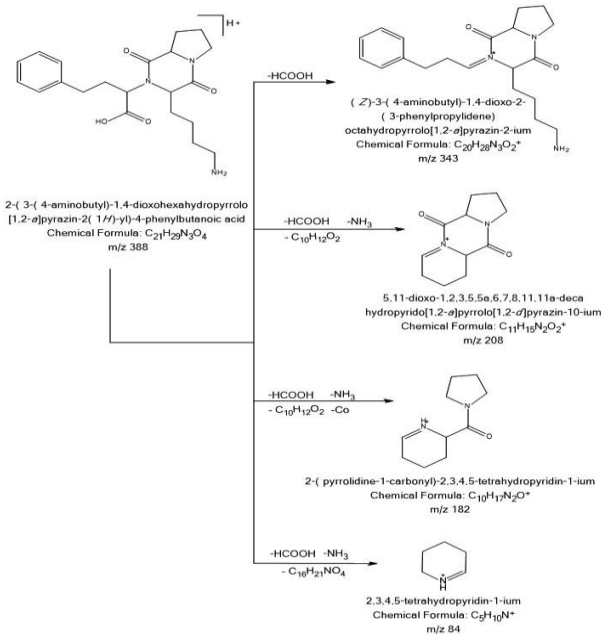

Figure 8. Possible schematic mechanism of fragmentation pattern pathway of the product ion (m/z 388^+).

Table 5. Summary of chemical formulae, relative abundance, measured and exact mass of major ions observed in the product ion MS/MS spectrum of [M+ H] (m/z 309^+).

Chemical formulae	Relative Abundance	Measured mass m/z [H+1]	Exact mass m/z [H+1]	Difference between exact and measured mass
$C_{16}H_{24}N_2O_4$	34.14 ±SD 0.08	309.375	309.174	0.201
$C_{15}H_{20}NO_2^+$	58.54 ±SD 0.36	246.572	246.148	0.424
$C_{14}H_{18}N^+$	17.27 ±SD 0.31	200.442	200.143	0.299
$C_7H_{12}NO_2^+$	27.91 ±SD 0.24	142.291	142.086	0.205
$C_5H_{18}N^+$	91.96 ±SD 0.15	84.579	84.081	0.498

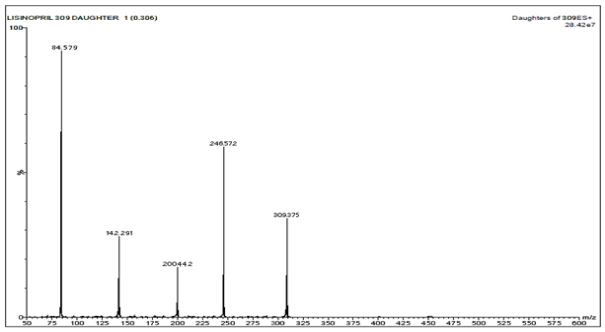

Figure 9. MS/MS spectrum of product ion [M+1] $C_{16}H_{24}N_2O_4$ at m/z 309$^+$.

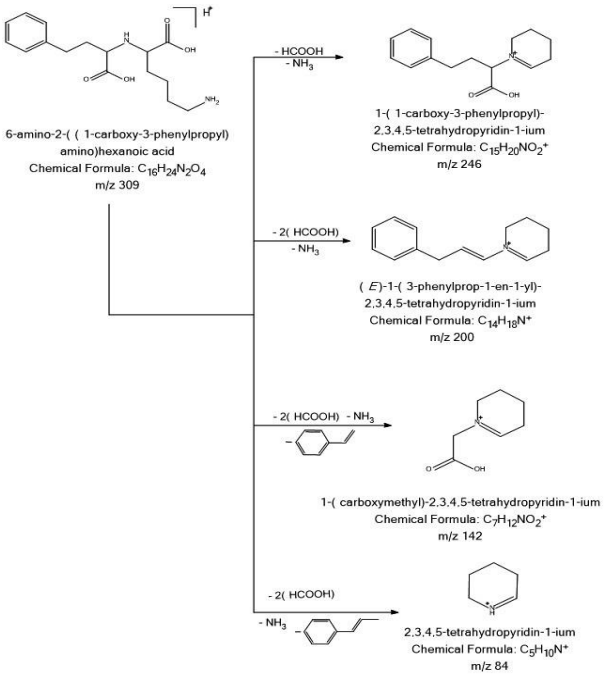

Figure 10. Possible schematic mechanism of fragmentation pattern pathway of the product ion (m/z 309$^+$).

The fragmentation ions produced by the protonated degradation product (m/z 244$^+$) was represented in **Figure 11**. It resulted from hydrolysis of lisinopril and elimination of the 4-phenylbutanoic acid. According to **Figure 12**, the fragmentation pattern of (m/z 244$^+$) under CID energy of 30.2v shows peaks at (m/z 225$^+$) due to elimination of the amino group and cyclization of the lysine residue to form the tetrahydropyridine ring, (m/z 179$^+$) due to the same pattern of (m/z 225$^+$) in addition to elimination of formic acid and formation of dihydropyrrole[19], (m/z 114$^+$) due to elimination of the 2,6-diaminohexanoyl molecule, and (m/z 84$^+$) due to the same pattern of (m/z 225$^+$) with the elimination of carboxypyrrolidine-1-carbonyl resulting in the formation of tetrahydropyridine moiety as presented in **Table 6**.

Table 6. Summary of chemical formulae, relative abundance, measured and exact mass of the major ions observed in the product ion MS/MS spectrum of [M+ H] (m/z 244$^+$).

Chemical formulae	Relative Abundance	Measured mass m/z [H+1]	Exact mass m/z [H+1]	Difference between exact and measured mass
$C_{11}H_{21}N_3O_3$	58.83 ±SD 0.04	244.492	244.158	0.334
$C_{11}H_{17}N_2O_3^+$	15.46 ±SD 0.18	225.291	225.123	0.168
$C_{10}H15_5N_2O^+$	42.97 ±SD 0.12	179.324	179.118	0.206
$C_5H_8NO_2^+$	29.91 ±SD 0.43	114.267	114.055	0.212
$C_5H_{10}N^+$	86.14 ±SD 0.38	84.572	84.081	0.491

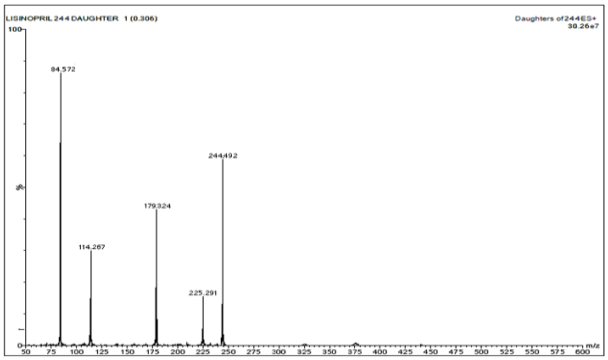

Figure11. MS/MS spectrum of the product ion [M+1] $C_{11}H_{21}N_3O_3$ at m/z 244$^+$.

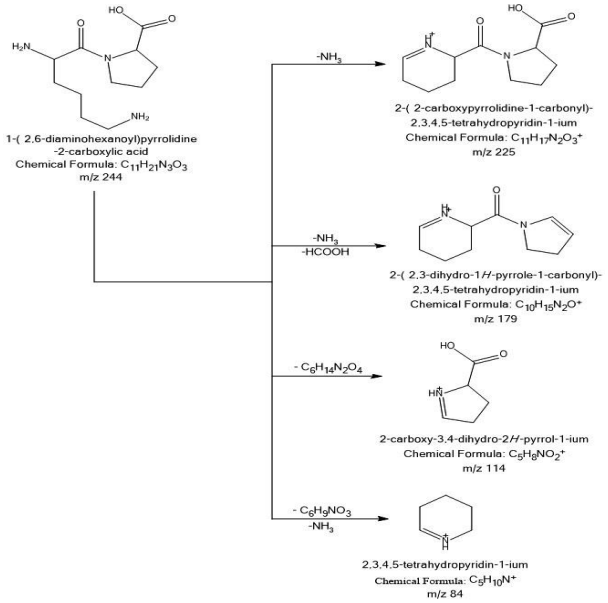

Figure 12. Possible schematic mechanism of fragmentation pattern pathway of the product ion (m/z 244$^+$).

Figure 13 shows the fragmentation ion produced from the protonated degradation product (m/z 180$^+$) by MS/MS. The protonated degradation product (m/z 180$^+$) resulted from hydrolysis of lisinopril to give 2-amino-4-phenylbutonic acid due to removing 6-aminohexanoyl pyrrolidine-2-carboxylic acid. The fragmentation pattern of (m/z 180$^+$) under CID energy of 42.86vshows peaks at (m/z 162$^+$) due to elimination of a water molecule, (m/z 134$^+$) due to elimination of formic acid, (m/z 105$^+$) due to elimination of 2-aminoacetic acid, and (m/z 72$^+$) due to elimination of water and toluene molecules as represented in **Table 7** and **Figure 14**.

Table 7. Summary of chemical formulae, relative abundance, measured and exact mass of major ions observed in the product ion MS/MS spectrum of [M+ H] (m/z 180$^+$)

Chemical formulae	Relative Abundance	Measured mass m/z [H+1]	Exact mass m/z [H+1]	Difference between the exact and measured mass
$C_{10}H_{13}NO_2$	38.76 ±SD 0.34	180.716	180.095	0.621
$C_{10}H_{11}NO$	22.49 ±SD 0.51	162.424	162.084	0.34
$C_9H_{11}N$	84.73 ±SD 0.42	134.393	134.089	0.304
C_8H_8	11.45 ±SD 0.33	105.279	105.063	0.216
C_3H_5NO	8.03 ±SD 0.47	72.346	72.037	0.309

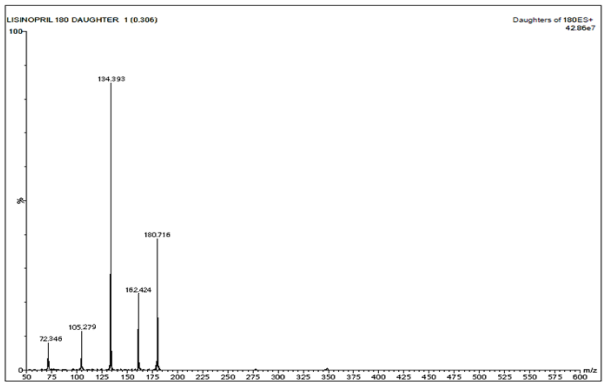

Figure 13. MS/MS spectrum of product ion [M+1] $C_{10}H_{13}NO_2$ at m/z (180$^+$).

From the previous studying of the fragmentation pattern pathways and schematic analysis of the degradation products resulted from the acidic hydrolysis of lisinopril, it was found that four main degradation compounds were formed as summarized in the **Table 8**. Both degradation products of m/z (388$^+$ and 180$^+$) were described in Eur. Ph. (8th) as lisinopril impurities[10].

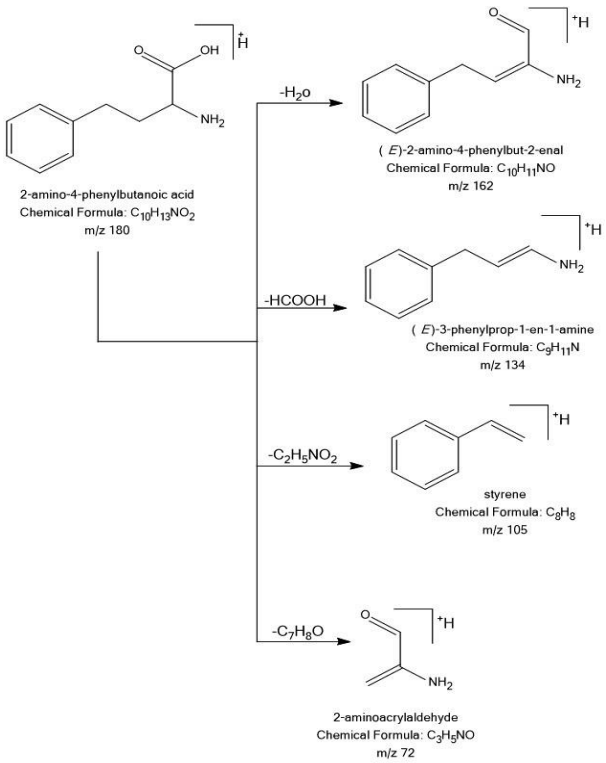

Figure 14. Possible schematic mechanism of fragmentation pattern pathway of the product ion (m/z 180$^+$).

Table 8. Formed degradation products resulted from acidic hydrolysis of lisinopril under stress condition.

Measured mass [M+1]	Relative Abundance	Chemical formulae	Chemical name
406$^+$	86.14 ±SD 0.11	$C_{21}H_{31}N_3O_5$	1-(6-amino-2-((1-carboxy-3-phenylpropyl)amino)hexanoyl)pyrrolidine-2-carboxylic acid [Lisinopril]
388$^+$	20.68 ±SD 0.11	$C_{21}H_{29}N_3O_3$	2-(3-(4-aminobutyl)-1,4-dioxohexahydropyrrolo[1,2-a]pyrazin-2(1H)-yl)-4-phenylbutanoic acid
309$^+$	25.5 ±SD 0.11	$C_{16}H_{24}N_2O_4$	6-amino-2-((1-carboxy-3-phenylpropyl)amino)hexanoic acid
244$^+$	55.42 ±SD 0.11	$C_{11}H_{21}N_3O_3$	1-(2,6-diaminohexanoyl)pyrrolidine-2-carboxylic acid
180$^+$	15.26 ±SD 0.11	$C_{10}H_{13}NO_2$	2-amino-4-phenylbutanoic acid

CONCLUSION

Four degradation products were resulted from acidic hydrolysis of lisinopril under drastic conditions. The formed degradation products were separated and detected by means of LC-MS/MS. The chemical structures of the degradation products were elucidated through studying the fragmentation pattern pathway of the fragmentation ions produced in MS/MS spectrum. Slight modification on the chromatographic conditions reported by Zhou to be applied for separation of lisinopril and degradation products was successively validated for determination of lisinopril by LC-MS/MS.

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