Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 538–545

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Stress degradation studies on lornoxicam using LC, LC–MS/TOF and LC–MS^n

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A R T I C L E I N F O

Article history:
Received 6 May 2011
Received in revised form 15 June 2011
Accepted 16 June 2011
Available online 23 June 2011

Keywords:
Lornoxicam
Stress testing
LC–MS
Characterization
Degradation mechanism

A B S T R A C T

Lornoxicam was subjected to forced degradation studies under hydrolytic (acidic, basic and neutral), oxidative, photolytic and thermal stress conditions, as defined under ICH guideline Q1A (R2). The drug degraded significantly in hydrolytic, oxidative and photoneutral conditions, leading to the formation of eight degradation products in total. It was stable on exposure to light and dry heat in the solid state. The stressed samples in which degradation was observed were mixed together and used to develop a stability-indicating HPLC method wherein degradation products were separated from the drug and also from each other. To characterize the degradation products, a complete mass fragmentation pathway of the drug was first established with the help of MS/TOF, MS^n and H/D exchange mass studies. The same was followed by LC–MS/TOF and on-line H/D exchange experiments on the degradation products. The degradation pathway of the drug was outlined, justified by the mechanisms of formation of the degradation products.

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1. Introduction

Hyphenated techniques, like liquid chromatography–mass spectrometry (LC–MS), have evolved as versatile tools for the characterization of drug impurities [1], degradation products [2], metabolites [3], environmental pollutants [4], food contaminants [5], etc.

In our laboratory, we have extensively employed LC–MS studies for the characterization of degradation products of drugs formed under various stress conditions [6–9]. Also, a systematic strategy has been reported for on-line characterization of degradation products using hyphenated LC–MS techniques [10]. In the present study, the same strategy was extended to study the degradation behavior of lornoxicam, a non-steroidal anti-inflammatory drug (6-chloro-4-hydroxy-2-methyl-*N*-pyridin-2-yl-2H-thieno[2,3-e][1,2]thiazine-3-carboxamide-1,1-dioxide; Fig. 1a). It acts by balanced COX-1/COX-2 enzyme inhibition [11,12]. The drug is not listed in any pharmacopoeia. A few reports on LC and LC–MS methods for the detection of lornoxicam and its metabolites in biological matrices are available [13,14], but very limited information exists on its decomposition behavior [15]. Neither the extent of degradation, nor the degradation pathways are reported.

2. Experimental

2.1. Drug and reagents

Lornoxicam was procured from Theon Pharmaceuticals Ltd. (Chandigarh, India). Hydrochloric acid, sodium hydroxide and hydrogen peroxide were procured from LOBA Chemie Pvt. Ltd. (Mumbai, India), Ranbaxy Laboratories (SAS Nagar, India) and s.d. Fine-chem Ltd. (Boisar, India), respectively. HPLC grade acetonitrile was purchased from J.T. Baker (Phillipsburg, NJ, USA). Buffer salts and all other chemicals were of analytical reagent grade. Ultrapure water was obtained from ELGA water purification unit (Bucks, England).

2.2. Apparatus and equipment

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for solution state degradation studies. A Dri-Bath (Thermolyne, IA, USA) was used for solid state thermal stress testing. Accelerated stability studies were carried out in a humidity chamber (KBF720, WTC Binder, Tuttlingen, Germany) set at 40 ± 1 °C/75 ± 3% RH. The photostability chamber (KBWF 240, WTC Binder) was equipped with an illumination bank on inside top, consisting of a combination of two UV (OSRAM L18W/73) and four white fluorescent (PHILIPS TRULITE 18W/86) lamps, in accordance with Option 2 of the ICH guideline Q1B [16]. Both fluorescent and UV lamps were put on simultaneously. The samples were placed at a distance of 0.23 m from the light bank. A lux meter (model ELM 201, Escorp, New Delhi, India) and UV-A

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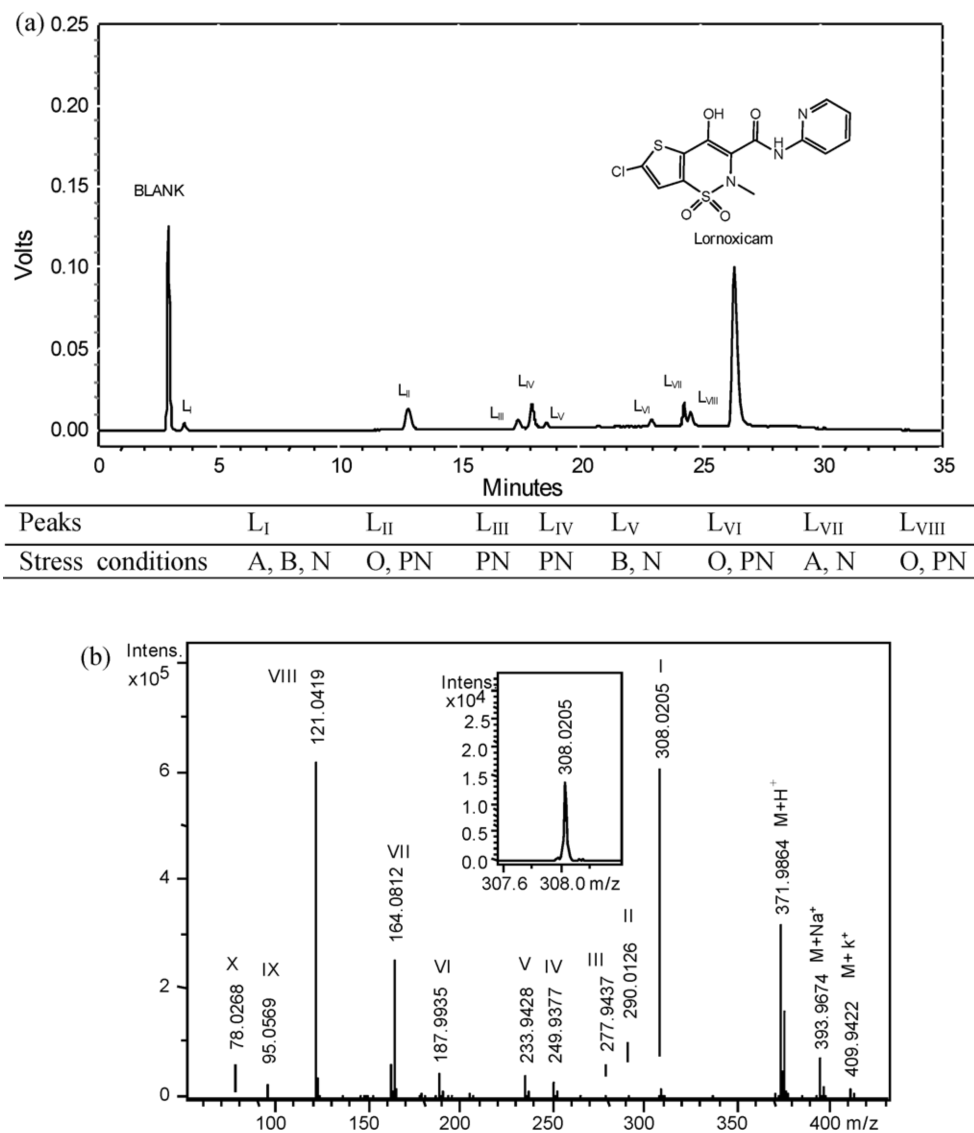
doi:10.1016/j.jpba.2011.06.012



Peaks	L_I	L_{II}	L_{III}	L_{IV}	L_V	L_{VI}	L_{VII}	L_{VIII}
Stress conditions	A, B, N	O, PN	PN	PN	B, N	O, PN	A, N	O, PN

Fig. 1. (a) Chromatogram showing separation of lornoxicam from its degradation products in the mixture of stressed samples and (b) MS/TOF line spectrum of lornoxicam. Key: $L_{I–VIII}$, degradation products of lornoxicam; A, acid; B, base; N, neutral; O, oxidative; PN, photo-neutral; I–X, fragments.

radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany) were used to measure visible illumination and UV-A energy, respectively. Two HPLC systems were used, one equipped with UV–visible dual-wavelength detector for method development and another with photo-diode array (PDA) detector for peak purity assessment. All other modules were same. The components of the two systems were on-line degasser (DGU-14A), low-pressure gradient flow control valve (FCV-10ALVP), solvent delivery module (LC-10ATVP), auto-injector (SIL-10ADVP), column oven (CTO-10ASVP), UV–visible dual-wavelength detector (SPD-10AVP), PDA detector (SPD-M10AVP), system controller (SCL-10AVP) and a computer system loaded with CLASS VP software (all from Shimadzu, Kyoto, Japan). Multi-stage MS (MS^n) studies were carried out on LTQ XL MS 2.5.0 (Thermo, San Jose, USA). The same was controlled by Xcalibur (version 2.0.7 SP1) software. LC–MS/TOF studies were carried out on a system in which the LC part consisted of 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The MS part consisted of MicrOTOF-Q spectrometer (Bruker Daltonik, Bremen, Germany), which was operated using Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. The TOF instrument was also used for H/D exchange study on the drug, while MS^n system was employed for on-line H/D exchange investigations on the degradation products. The calibrant used was 5 mM solution of sodium formate (Sigma–Aldrich, New Delhi, India). All masses were corrected by internal reference ions of m/z 90.9766, 158.9640, 226.9514, 294.9389, 362.9263, 430.9137, 490.9011, 566.8866 and 634.8760. The LC separation was achieved on an Inertsil C-18 (250 mm × 4.6 mm i.d., particle size 5 μm) column (LCGC Chromatography Solution, Hyderabad, India).

2.3. Stress studies

Stress studies were conducted on the drug in hydrolytic, oxidative, photolytic and thermal conditions using the protocol developed in our laboratory [17]. The drug was partially soluble in water; hence a mixture of ACN and water (50:50) was employed for the preparation of stock solution, which contained 2 mg/ml of the drug. The same was diluted with an equal volume of the stressor. Hydrolytic stress studies were carried out at 80 °C in acidic, alkaline and neutral media using hydrochloric acid (0.1 N), sodium hydroxide (0.5 N) and water, respectively. Oxidative degradation study was carried out in hydrogen peroxide (3% v/v) at room temperature.

Table 1
Optimized stress conditions for the drug.

Stressors	Hydrolytic at 80 °C			Oxidative at RT	Photolytic at 8500 Lux fluorescent and 0.5 W h/m^2 UV light at 40 °C/75% RH				Thermal at 50 °C
	Acid	Neutral	Base		Acid	Neutral	Base	Solid	
Concentration of stressor	0.1 N HCl	H_2O	0.1 N NaOH	3% H_2O_2	0.01 N HCl	H_2O	0.01 N NaOH	–	–
Duration of exposure	3 h	3 h	1 h	36 h	7 d	2 d	7 d	10 d	21 d

Table 2
MS/TOF and H/D exchange data of the drug.

Peak no.	Experimental mass	Best possible molecular formula[a]	Theoretical mass	Error (mmu)	RDB	Possible parent fragment	Difference from parent ion	Possible losses corresponding to difference		H/D Exchange data	No. of labile hydrogens
								L1	L2		
M+H⁺	371.9864	$C_{13}H_{11}N_3O_4S_2Cl$	371.9874	−1.0	9.5					374	2
I	308.0205	$C_{13}H_{11}N_3O_2SCl$	308.0255	−5.0	9.5	M+H⁺	63.9659	SO_2		310	2
II	290.0126	$C_{13}H_9N_3OSCl$	290.0149	−2.3	10.5	I	18.0079	H_2O		291	1
III	277.9437	$C_8H_5NO_4S_2Cl$	277.9343	9.4	6.5	M+H⁺	94.0427	$C_5H_6N_2$	C_6H_6O	279	1
IV	249.9377	$C_7H_5NO_3S_2Cl$	249.9394	−1.7	5.5	III	28.0060	CO		251	1
V	233.9428	$C_7H_5NO_2S_2Cl$	233.9445	−1.7	5.5	IV	15.9949	H_2O		234	0
VI	187.9935	C_7H_7NOSCl	187.9931	0.4	4.5	I	120.0270	$C_6H_4N_2O$	$C_6H_4N_2O$	189	2
VII	164.0812	$C_8H_{10}N_3O$	164.0818	−0.6	5.5	M+H⁺	207.9050	$C_5HS_2ClO_3$		166	2
VIII	121.0419	$C_6H_5N_2O$	121.0396	2.3	5.5	VII	43.0393	C_2H_5N	CH_3N_2	122	1
IX	95.0569	$C_5H_7N_2$	95.0604	−3.5	3.5	VIII	25.9850	CO		97	2
X	78.0268	C_5H_4N	78.0338	−7.0	4.5	IX	17.0301	NH_3		78	0

[a] Determined from experimental accurate mass using elemental composition calculator.

Photolytic studies were conducted in solution (acid, alkali and neutral) as well as in solid state. For thermal stress study, the drug was sealed in glass vials and placed in a thermostatic block at 50 °C. The optimized stressed conditions for the drug are listed in Table 1.

2.4. HPLC method development and optimization

The stressed samples were withdrawn at suitable time intervals and diluted four times with the solvent before analysis by HPLC. Also, all the stressed samples were mixed together in an equal volume and the mixture was used for HPLC method development. A gradient method was developed by varying the ratio of methanol and potassium dihydrogen phosphate buffer to get sufficient resolution between the drug and its degradation products.

2.5. MS/TOF, MS^n and H/D exchange studies on the drug

Separate direct injection mass methods were developed on MS/TOF in ESI positive mode to get the molecular ion peak and fragmentation pattern of the drug. Mass parameters were optimized to the following values: end plate offset, −500 V; capillary voltage, −4500 V; nebuliser gas pressure, 1.2 bar; dry gas flow, 6.0 l/min; dry temperature, 200 °C; funnel 1 RF, 250 Vpp; funnel 2 RF, 280 Vpp; ISCID energy, 0.0 eV; hexapole RF, 300 Vpp; quadrupole ion energy, 5.0 eV/z; collision energy, 5.0 eV/z; transfer time, 45 μs; collision RF, 240 Vpp; pre-pulse storage, 7.0 μs. Further, funnel 1 RF, hexapole RF, collision energy, transfer time and collision RF were modified to 240 Vpp, 280 Vpp, 12.0 eV/z, 55 μs and 190 Vpp, respectively, to get complete fragmentation of the drug.

2.6. LC–MS/TOF and on-line H/D exchange studies on degradation products

For characterization of the degradation products by LC–MS, the developed HPLC method was modified wherein phosphate buffer was replaced by ammonium formate buffer of the same pH and molarity. While most degradation products of the drug were observed in ESI positive mode, one of them appeared in ESI negative mode. The MS/TOF parameters were optimized to get molecular ion and fragments of all the degradation products and were almost same as for the drug. The drug was also subjected to MS^n and H/D exchange studies to find out the origin of each fragment, and the number of labile hydrogens present in the structure, respectively. On-line H/D exchange mass studies were carried out using the LTQ-MS^n system through injection of D_2O via an additional channel, just before the peak of interest started eluting from the column. The flow of D_2O was continued until the peak was completely eluted. All the degradation products were then characterized on the basis of data obtained from exact mass, fragmentation pattern and on-line H/D exchange studies.

3. Results and discussion

3.1. Optimized HPLC method and chromatographic separation

The finally optimized stability-indicating HPLC method was developed on a C-18 column using a mobile phase comprising of potassium dihydrogen phosphate buffer (10 mM, pH 3.3) and methanol. Methanol (A) and buffer (B) were varied in a gradient program (T_{min}/A:B; $T_{0.01}$/10:90; $T_{1.0}$/10:90; $T_{10.0}$/43:57; $T_{16.0}$/65:35; $T_{20.0}$/65:35; $T_{35.0}$/10:90). The detection wavelength was 254 nm and the flow rate was 1.0 ml/min. The chromatogram showing resolution of the drug and its degradation products in a mixture of stressed solutions is shown in Fig. 1a.

Table 3
MS^n fragmentation of the drug.

MS^n	Precursor ion	Product ions
MS^2	371	308, 277, 164
MS^3	308	290, 187
	277	249
	164	121
MS^4	249	233
	121	95
MS^5	95	78

Table 4
MS/TOF and on-line H/D exchange data of degradation products of lornoxicam ($L_{I–VIII}$) along with the best possible molecular formulae and number of labile hydrogens.

Degradation product	ESI mode	Experimental mass (amu)	Molecular formulae (exact mass; RDB; error in mmu)	Mass after H/D exchange	Number of labile hydrogens
L_I	Positive	95.0538	$C_5H_7N_2^+$ (95.0604; 3.5; −6.6)	98	2
L_{II}	Positive	167.0503	$C_7H_7N_2O_3^+$ (167.0451; 5.5; 5.2)	170	2
L_{III}	Positive	180.0760	$C_8H_{10}N_3O_2^+$ (180.0768; 5.5; −0.8)	183	2
L_{IV}	Negative	240.9024	$C_5H_2ClO_5S_2^-$ (240.9028; 4.5; −0.4)	241	2
L_V	Positive	226.9203	$C_5H_4ClO_4S_2^+$ (226.9234; 3.5; −3.1)	228	1
L_{VI}	Positive	255.9523	$C_6H_7ClNO_4S_2^+$ (255.9500; 3.5; 2.3)	258	2
L_{VII}	Positive	251.9531	$C_7H_7ClO_3S_2^+$ (251.9550; 4.5; −1.9)	253	1
L_{VIII}	Positive	237.9379	$C_6H_5ClNO_3S_2^+$ (237.9394; 4.5; −1.5)	238	0

3.2. Degradation behavior of the drug

Lornoxicam was observed to be susceptible to hydrolytic and oxidative conditions. Its solution in water was also labile to light. However, the drug was stable to thermal stress and to light in solid state. As shown in Fig. 1a, stress studies on the drug resulted in a total of eight degradation products (labeled L_I–L_{VIII}). The product L_I was formed in all hydrolytic conditions; L_V in both basic and neutral conditions, and L_{VII} in acidic as well as neutral conditions. The degradation products L_{II}, L_{VI} and L_{VIII} were formed in oxidative solution, while L_{II}–L_{IV}, L_{VI} and L_{VIII} were observed on exposure of neutral solution of the drug to light.

3.3. Mass fragmentation pathway of the drug

The mass fragmentation pathway of the drug was established from results of MS/TOF, MS^n and H/D exchange studies, using optimized mass parameters. The MS/TOF spectrum of the drug is shown in Fig. 1b, which depicts a total of ten fragments (labeled 'I–X'), apart from Na and K adducts. The molecular formulae of the fragments and possible losses were determined using an elemental composition calculator (Table 2). The origin of each fragment was established through MS^n studies (Table 3).

The postulated pathway is outlined in Fig. 2. According to the same, the drug ionized ($M+H^+$, m/z 372) at two different possible sites involving sulfonamido and carboxamido nitrogens and fragmented in MS^2 step into daughters of m/z 308 (I), 278 (III) and 164 (VII). The drug lost SO_2 from the sulfonyl moiety to result in a fragment of m/z 308 (I). The latter further yielded fragments of m/z 290 (II) and 188 (VI). The ion of m/z 278 (III) was generated upon protonation of carboxamido nitrogen and cleavage of C–N bond. In MS^3 step, it fragmented into daughter of m/z 250 (IV) involving neutral loss of CO and the same further converted in MS^4 step into fragment of m/z 234 (V) on the loss of H_2O molecule. The product ion of m/z 164 (VII) was a result of lysis of the S–N bond of the drug protonated at sulfonamido group. It fragmented further by following the sequence m/z 164 → m/z 121 (VIII) → m/z 95 (IX) → m/z 78 (X) on the loss of C_2H_4NH, CO and NH_3 moieties in MS^3, MS^4 and MS^5 steps, respectively. The structures in Fig. 2 were supported by H/D exchange data and calculated ring plus double bond (RDB) values given in Table 2.

3.4. LC/MS–TOF and on-line H/D exchange studies on the stressed samples

The LC–MS/TOF spectra of the degradation products generated in stressed samples are shown in Fig. 3. Their experimental masses, best possible molecular formulae and number of labile hydrogens are listed in Table 4.

3.5. Postulated structures of the degradation products

The degradation products were characterized systematically with the help of HRMS, mass fragmentation and on-line H/D exchange data, using the strategy outlined by Mehta et al. [10]. The proposed structures of all the degradation products are overlaid on mass spectra in Fig. 3.

3.5.1. L_I (m/z 95.0538)

The experimental accurate mass of L_1 was 95.0538 Da and it reduced to single fragment of 78.0321 (Fig. 3), indicating loss of NH_3. As shown in Fig. 2, the same mass parent (95 Da) and product (78 Da) were also observed in the fragmentation pathway of the drug. Accordingly, the product was identified as 2-aminopyridine. The same was justified by molecular formula predicted by elemental composition calculator ($C_5H_7N_2^+$) and shift of molecular ion peak to 98 Da on on-line H/D exchange, indicative of the presence of two labile hydrogens in the structure.

3.5.2. L_{II} (m/z 167.0503)

The HRMS value of L_{II} was 167.0503 Da. The elemental composition calculator suggested $C_7H_7N_2O_3^+$ as the molecular formula (theoretical mass 167.0451 Da). H/D exchange studies showed the possibility of two labile hydrogens in the structure. On fragmentation, it yielded ions of m/z 121 and m/z 123 upon the loss of H_2CO_2 and CO_2, respectively. The mass of the ion of m/z 121 was found to match with that observed in the drug. Accordingly, identity of the product was established to be 2-oxo-2-(pyridin-2-ylamino)acetic acid.

3.5.3. L_{III} (m/z 180.0760)

For L_{III}, elemental composition calculator suggested $C_8H_{10}N_3O_2^+$ as the most probable molecular formula. The error between its experimental (180.0760 Da) and theoretical mass (180.0768) was just −0.8 mmu. On fragmentation, it gave fragments of m/z 149 and m/z 121 on sequential loss of CH_3NH_2 and CO, respectively. In this case also, the mass of ion of m/z 121 was same as that for the drug, revealing that the product originated from the pyridinyl part of the drug. In addition to the above, the presence of two labile hydrogens in the structure (indicated through on-line H/D exchange studies) helped in proposing the degradation product to be N^1-methyl-N^2-(pyridin-2-yl)oxalamide.

3.5.4. L_{IV} (m/z 240.9024)

This product was exclusively seen in ESI negative mode and had accurate experimental mass of 240.9024 Da. The theoretical mass for the same was 240.9032, corresponding to the molecular formula of $C_5H_2ClO_5S_2^-$ (error = −0.8 mmu). On-line H/D exchange data showed possibility of single labile hydrogen in the molecular ion. This along with poor ionization in positive mode indicated a free carboxylic group and absence of nitrogen in the structure,

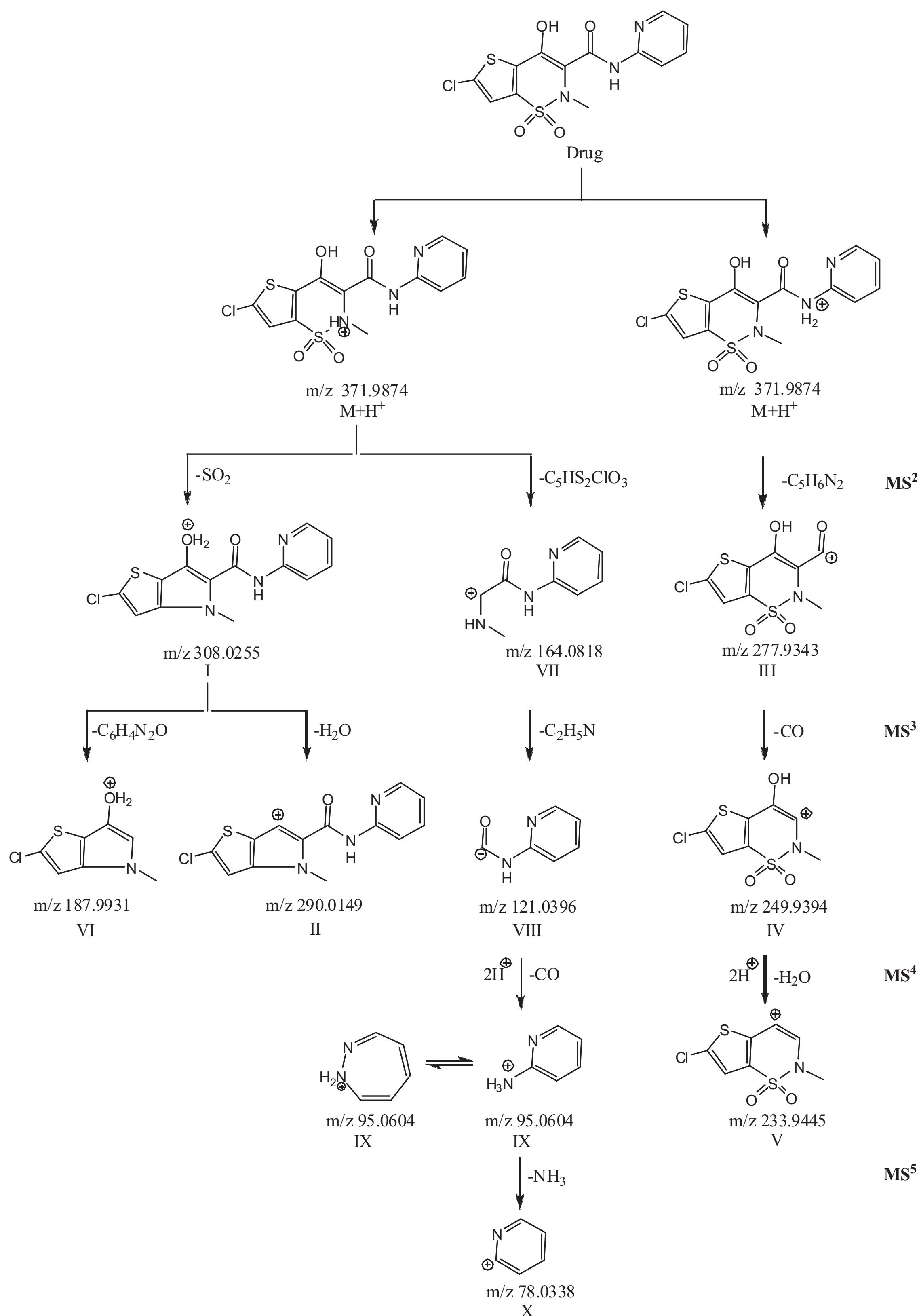


Fig. 2. Fragmentation pathway of the drug along with the exact masses of the fragments.

which was substantiated by appearance of a fragment involving neutral loss of 43.9901 Da corresponding to CO_2. Based on these observations and also considering the isotopic ratio, the product was established as 5-chloro-3-sulfothiophene-2-carboxylic acid.

3.5.5. L_V (m/z 226.9203)

The experimental accurate mass of this product was determined to be 226.9203 Da in ESI positive mode. Its formula was suggested through elemental composition calculator to be $C_5H_4ClO_4S_2^+$ (theoretical exact mass 226.9234 Da, error = −3.1 mmu). It showed

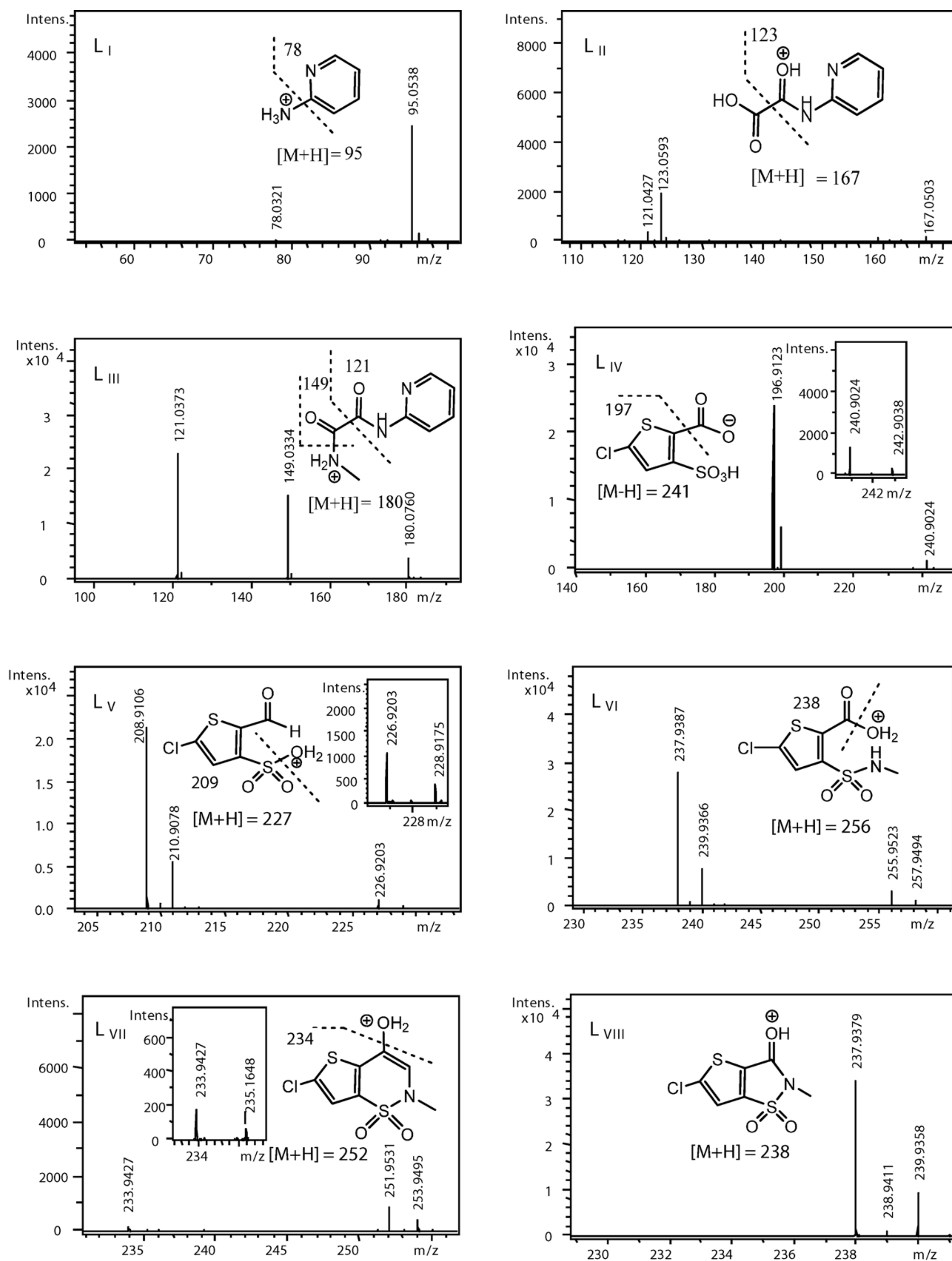


Fig. 3. Structures and mass fragmentation spectra of the degradation products of lornoxicam (L_I, L_{II}, L_{III}, L_V, L_{VI}, L_{VII} and L_{VIII}) in ESI positive mode and L_{IV} in ESI negative mode.

major fragment of *m*/*z* 209 with a difference of 18 Da, suggesting loss of water from the structure. The presence of chlorine atom in the molecular ion peak and even its fragment was confirmed by isotopic abundance (M+2) ratio analysis. On-line H/D exchange experiment indicated that the structure had single labile hydrogen. These observations were found to be consistent with 5-chloro-2-formylthiophene-3-sulfonic acid as the structure of the degradation product.

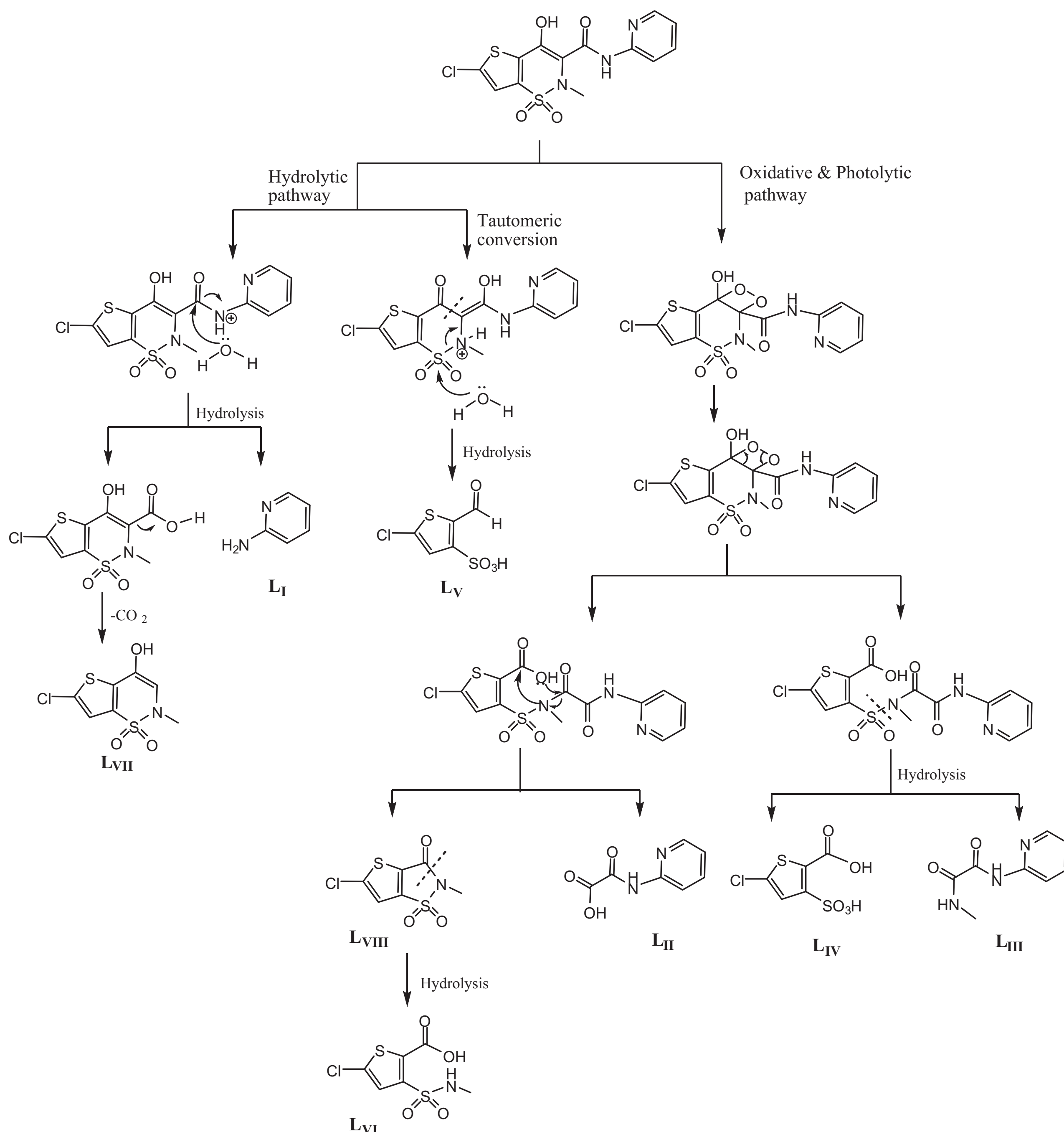


Fig. 4. Pathway and mechanisms for the decomposition of lornoxicam in hydrolytic, oxidative and photolytic conditions.

3.5.6. L_{VI} (m/z 255.9523)

The experimental accurate mass of L_{VI} was 255.9523 Da. The elemental composition calculator suggested the molecular formula to be $C_6H_7ClNO_4S_2^+$ (theoretical exact mass 255.9500, error = 2.3 mmu). The M+2 isotopic peak appeared at *m/z* 258 and was about 32% of the product confirming the presence of chlorine atom. The same isotopic behavior was seen even for the fragment that appeared at *m/z* 238 on the loss of water from the parent. The degradation product conforming to these observations was identified as 5-chloro-3-(*N*-methylsulfamoyl)thiophene-2-carboxylic acid.

3.5.7. L_{VII} (m/z 251.9531)

The product had accurate mass value of *m/z* 251.9531 and its predicted molecular formula was $C_7H_7ClO_3S_2^+$ (exact mass 251.9550 Da, error = −1.9 mmu). The isotopic behavior of parent and daughter peaks confirmed that it was also a chlorinated product. The fragment appeared at *m/z* 234 indicating loss of water from the parent. On line H/D exchange studies showed possibility of single labile hydrogen. The structure justifying these observations was postulated as 6-chloro-4-hydroxy-2-methyl-2H-thieno[2,3-e]-1,2-thiazine-1,1-dioxide.

3.5.8. L_{VIII} (m/z 237.9379)

The product L_{VIII} had an experimental accurate mass of 237.9379 Da. Its molecular formula was $C_6H_5ClNO_3S_2^+$ and the difference between experimental mass from theoretical mass (237.9394 Da) was just −1.5 mmu. An isotopic peak of *m/z* 240 indicated the presence of chlorine in the structure. Incidentally, no

fragment peak was observed in this case. On-line H/D exchange studies confirmed the absence of any labile hydrogen in the structure. The structure of the product justifying the above was postulated to be 5-chloro-2-methyl-thieno[2,3-d]-isothiazolidin-3-one-1,1-dioxide.

3.6. Degradation pathways and mechanism

The structures of degradation products showed that the drug degraded primarily by amide hydrolysis and oxidative/photooxidative decomposition. The hydrolysis occurred at carboxamide bond to form the products L_I and L_{VII}. The attack of water on sulfonamide bond resulted in L_V. In oxidative and photolytic conditions, the drug underwent autoxidation at enolic double bond and further intramolecular nucleophilic attack to yield L_{II}, and L_{VIII}. A parallel hydrolytic reaction formed L_{III} and L_{IV}. The product L_{VIII} sequentially got hydrolyzed into L_{VI} [18,19]. The degradation pathway along with mechanisms of formation of all the degradation products is shown in Fig. 4.

4. Conclusion

A stress degradation study was performed on lornoxicam. The investigation provided fruitful information regarding degradation behavior of the drug under conditions of hydrolysis, oxidation, photolysis and thermal stress. The structures of all the degradation products were delineated by comparison of the accurate mass, fragmentation pattern, molecular formulae, RDB and H/D exchange data. The same were supported by appropriate mechanisms of their formation. The comprehensive mass fragmentation profile was established for lornoxicam, which has not been reported so far. Amide hydrolysis and oxygen addition across the enolic double bond were found to be the major degradation routes of the drug.

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