Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 55–63

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Study of forced decomposition behavior of lamivudine using LC, LC–MS/TOF and MS^n

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ARTICLE INFO

Article history:
Received 22 September 2008
Accepted 4 October 2008
Available online 15 October 2008

Keywords:
Lamivudine
Stress studies
Stability-indicating assay method
MS^n
LC–MS/TOF
Degradation pathway
Mechanism

ABSTRACT

Lamivudine was subjected to forced decomposition conditions of hydrolysis (neutral, acidic and alkaline), oxidation, photolysis and thermal stress, as suggested in the ICH guideline Q1A(R2). The drug showed instability in acid and alkali, while it remained stable in neutral conditions. It also degraded extensively under oxidative environment. It remained stable to light and thermal stress. In total, five degradation products were formed, which could be separated by LC on a C18 column using a gradient method. To characterize the products, first a complete fragmentation pathway of the drug was established by carrying out multi-stage (MS^n) and MS/TOF accurate mass studies. The same was compared to fragment pattern of the degradation products resulting from LC–MS/TOF studies. The accurate mass values obtained from LC–MS/TOF were used to obtain elemental compositions, and the total information helped in identification of the degradation products. Subsequently, degradation pathway of the drug was laid down, along with mechanisms of formation of the degradation products. There is no previous information on these aspects on the drug in the literature.

1. Introduction

Lamivudine belongs to the class of dideoxynucleoside reverse transcriptase inhibitors, and is a potent inhibitor of human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS) [1,2]. Intracellularly, lamivudine is phosphorylated to an active 5′-triphosphate metabolite. Lamivudine triphosphate inhibits the activity of HIV-1 reverse transcriptase by DNA chain termination after incorporation of the nucleoside analogue into viral DNA [3]. Furthermore, lamivudine also shows activity against hepatitis B virus [4].

Chemically, lamivudine is (2*R*,5*S*)-4-amino-1-(2-hydroxymethyl-1,3-oxathiolan-5-yl)-(1*H*)-pyrimidin-2-one (Fig. 1). In the literature, many LC and LC–MS methods have been reported for analysis of the drug and its metabolites in biological fluids [5–9]. However, very little is reported on the decomposition behavior of the drug. Kaul et al. carried out stress studies on the drug under acidic, basic, oxidative, thermal and photolytic conditions, and separated the products on a HPTLC plate [10]. But neither the extent of degradation, nor the nature/structures of degradation products, and the degradation pathway were reported. A monograph on the drug was finalized in 2006 by the World Health Organization (WHO) for inclusion in the *International Pharmacopoeia* (Ph. Int.), in which a list of 12 impurities and their structures were provided [11]. However, the listed impurities are not classified into process impurities and degradation products. So the intrinsic degradation profile of drug under prescribed stress conditions [12,13] is still unknown.

Hence, an integral aim of the present study was to investigate the complete degradation behavior of the drug. It was done through a systematic investigation involving: (i) forced decomposition of the drug under a variety of stress conditions, (ii) resolution of products employing a LC–MS compatible method, (iii) conduct of LC–MS studies to establish fragmentation profiles of the drug and the degradation products, (iv) elucidation of structures of degradation products through comparative study of mass data, and (v) ascertaining degradation pathway and mechanism of decomposition of the drug based on the total information collected.

2. Experimental

2.1. Drug and reagents

Pure lamivudine was obtained as gratis sample from Aurobindo Pharma Ltd. (Hyderabad, India) and it was used without further purification. Analytical reagent (AR) grade sodium hydroxide (NaOH) was purchased from Ranbaxy Laboratories (SAS Nagar, India), hydrochloric acid (HCl) from LOBA Chemie Pvt. Ltd.

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doi:10.1016/j.jpba.2008.10.002

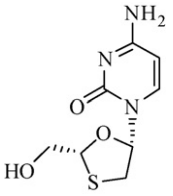


Fig. 1. Structure of lamivudine.

(Mumbai, India) and hydrogen peroxide (H_2O_2) from S.D. Fine-Chem Ltd. (Boisar, India). Buffer salts and all other chemicals were also of AR grade. HPLC grade acetonitrile (ACN) was procured from J.T. Baker (Mexico City, Mexico). Ultra pure water obtained from ELGA water purification unit (Wycombe, Bucks, England) was used throughout the studies.

2.2. Apparatus and equipment

Precision water baths equipped with MV controller (Julabo, Seelbach, Germany) were used for solution degradation studies. A Dri-Bath (Thermolyne, IA, USA) was used for solid state thermal stress studies. Accelerated stability studies were carried out in humidity (KBF720, WTC Binder, Tuttlingen, Germany) and photostability (KBWF 240, WTC Binder) chambers, both set at $40 \pm 1\,^{\circ}C/75 \pm 3\%$ RH. The photostability chamber was equipped with an illumination bank on inside top, consisting of a combination of two UV (OSRAM L18 W/73) and four white fluorescent (PHILIPS TRULITE 18W/86) lamps, in accordance with 'Option 2' of ICH guideline Q1B [14]. Both fluorescent and UV lamps were put on simultaneously. The samples were placed at a distance of 9 in. from the light bank. A calibrated lux meter (model ELM 201, Escorp, New Delhi, India) and a calibrated near UV radiometer (model 206, PRC Krochmann GmbH, Berlin, Germany) were used to measure visible illumination and near UV energy, respectively.

The HPLC system consisted of an 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), photo-diode array (PDA) detector (SPD-M10AVP), system controller (SCL-10AVP) and a computer system loaded with CLASS-VP software (all from Shimadzu, Kyoto, Japan). Multiple stage MS (MS^n) studies were carried out on LXQ linear ion-trap equipment (Finnigan Mat, San Jose, USA). The same was controlled by Xcalibur (ver. 2.0) software. LC–MS/TOF studies were carried out on a system in which LC part consisted of 1100 series HPLC from Agilent Technologies (Waldbronn, Germany). The MS part consisted of MicrOTOF-Q spectrometer (from Bruker Daltonik, Bremen, Germany), which was operated using Hyphenation Star (version 3.1) and MicrOTOF Control (version 2.0) software. In all the studies, separations were achieved on a C18 column (250 mm × 4.6 mm i.d., particle size 5 μm, Princeton Chromatography Inc., Cranbury, NJ, USA).

2.3. Forced degradation studies

Stress studies were carried out under ICH prescribed stress conditions [12], *viz.*, hydrolysis (acid, base and neutral), oxidation, photolysis and dry heat. The drug was subjected to forced degradation under acidic, basic and neutral conditions by refluxing in 0.1N HCl, 0.1N NaOH and water at 80 °C for 48 h, 12 h and 72 h, respectively. The drug concentration was 1 mg ml^{-1}. Oxidative stress studies were carried out at room temperature for 48 h in 3% H_2O_2 and 30% H_2O_2 at a concentration of 1 mg ml^{-1} and 10 mg ml^{-1}, respectively. For thermal stress, the drug was kept at 50 °C for 2 months. Photolytic studies were carried out on the drug in solid as well as in solution state. For solution state, the drug was dissolved in 0.1N HCl and 0.1N NaOH at a concentration of 1 mg ml^{-1} and exposed to 1.2×10^6 lx h of fluorescent light and 200 Wh/m^2 UV-A light in a photostability chamber. Solid drug was also exposed to the same dose of light in a thin layer (1 mm) in a petri-plate.

2.4. Preparation of samples for HPLC analyses

The reaction samples in water, 0.1N HCl, 0.1N NaOH and 3% H_2O_2 were diluted 10 times with water. The samples in 30% H_2O_2 were diluted 100 times with water. The solid samples were suitably diluted in water. All the solutions were filtered using 0.22 μm membrane filter before HPLC injections.

2.5. LC studies

The individual reaction solutions were initially subjected to analysis by a method involving use of a C18 column (5 μm) and a mobile phase comprising of a mixture of 5 volumes of methanol R and 95 volumes of buffer pH 3.8 (a 1.9-g/l solution of ammonium acetate R, previously adjusted to pH 3.8 with glacial acetic acid R). The other conditions were flow rate, 1.0 ml min^{-1}; detection wavelength, 277 nm; and temperature, 35 °C [11]. As the separation of drug and degradation products was not selective, therefore, logical changes, like pH, ratio of mobile phase components, flow rate and column temperature were tried. The optimized method was validated with respect to various parameters outlined in the ICH guideline Q2(R1) [15]. To establish linearity and range, a stock solution containing 1 mg ml^{-1} drug in water was diluted to yield solutions in the concentration range of 50–500 μg ml^{-1}. The solutions were prepared in triplicate and analyzed by injecting 10 μl into HPLC. The intra- and inter-day precision were established by analyzing 100 μg ml^{-1}, 300 μg ml^{-1} and 500 μg ml^{-1} drug solutions three times on the same day and on 3 consecutive days, respectively. To determine intermediate precision, the brand of the column was changed and also whole experiment was conducted by a different person. Accuracy was determined by spiking a mixture of stressed samples with three known concentrations of the drug, *viz.*, 100 μg ml^{-1}, 300 μg ml^{-1} and 500 μg ml^{-1} in triplicate and then determining the percent recovery of the added drug. Specificity of the method was established by determining purity

Table 1
Data for lamivudine from linearity studies.

Conc. (μg ml^{-1})	Conc. 1	Conc. 2	Conc. 3	Average	±S.D.	R.S.D. (%)
50	1,192,886	1,192,763	1,191,215	1,192,288	931.27	0.078
100	2,340,272	2,341,553	2,341,821	2,341,215	827.86	0.035
200	4,483,013	4,486,078	4,473,416	4,480,835	6605.84	0.147
300	6,843,537	6,848,598	6,852,106	6,848,080	4307.89	0.062
400	9,253,875	9,237,714	9,248,714	9,246,768	8254.43	0.089
500	11,351,633	11,369,949	11,363,012	11,361,531	9247.33	0.081

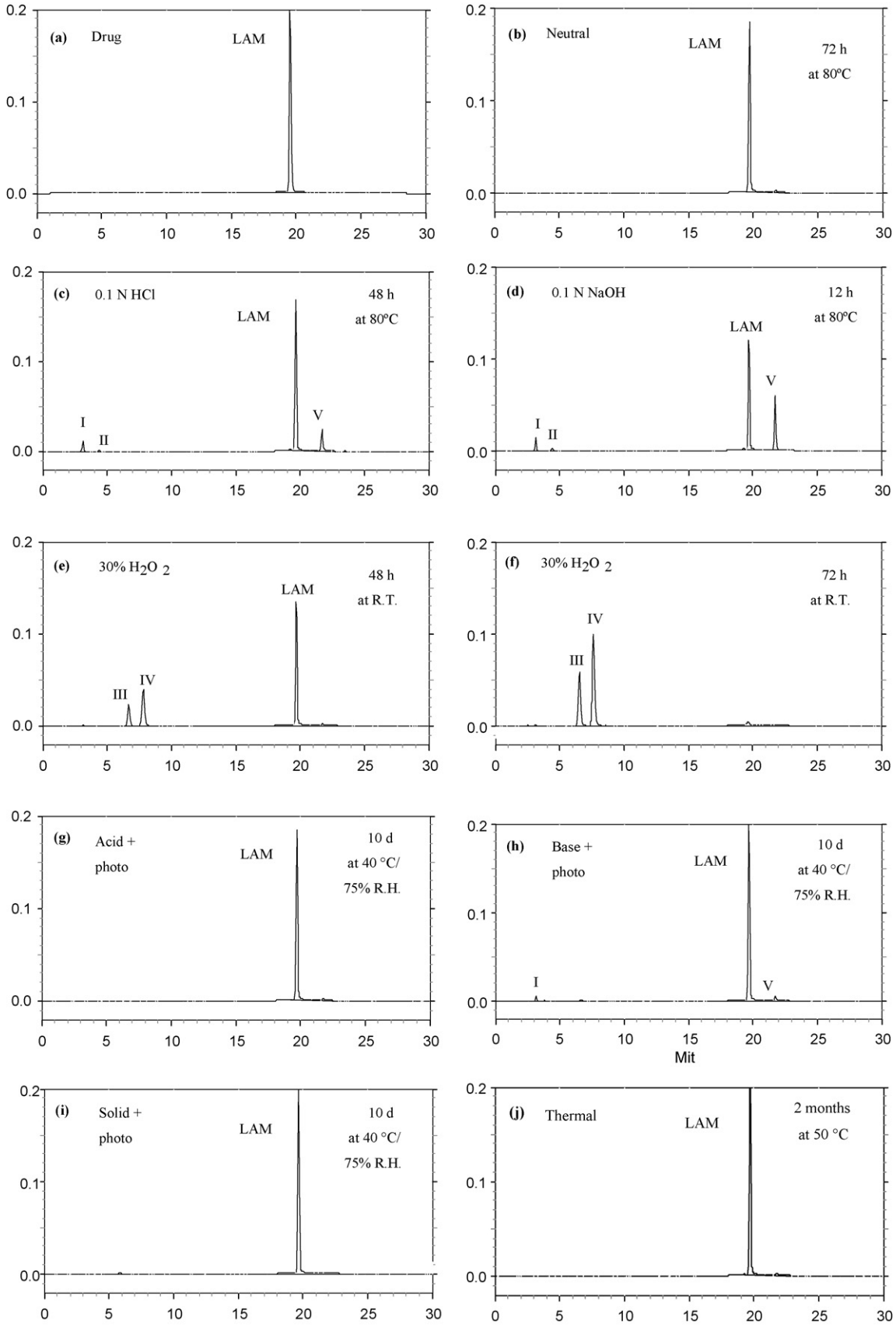


Fig. 2. Chromatograms showing lamivudine and its degradation under hydrolytic, oxidative and photolytic conditions (LAM: lamivudine; I–V degradation products; R.T.: room temperature).

Table 2
Peak area values for lamivudine from intra- and inter-day studies.

Conc. ($\mu g\,ml^{-1}$)	Intra-day precision Measured conc. ± S.D. ($\mu g\,ml^{-1}$), R.S.D. (%)	Inter-day precision Measured conc. ± S.D. ($\mu g\,ml^{-1}$), R.S.D. (%)
100	101.4147 ± 0.036, 0.035	101.4507 ± 0.081, 0.080
300	299.4838 ± 0.189, 0.063	300.6580 ± 0.141, 0.046
500	497.8424 ± 0.406, 0.081	498.7284 ± 0.879, 0.176

Table 3
Recovery data for lamivudine spiked into a mixture of stressed samples.

Spiked conc. ($\mu g\,ml^{-1}$)	Calculated spiked conc. ($\mu g\,ml^{-1}$) ± S.D., R.S.D. (%)	Recovery (%)
100	101.4021 ± 0.027, 0.026	101.40
300	299.5565 ± 0.149, 0.049	99.85
500	498.5922 ± 0.363, 0.072	99.71

of each peak using a PDA detector and evaluation of resolution factors.

2.6. MS^n and MS/TOF studies on the drug

The fragmentation profile of the drug was established by carrying out MS^n (up to MS^3) and MS/TOF studies in positive electrospray ionization (+ve ESI) mode. The mass parameters for MS^n studies were: spray voltage, 4.75 kV; capillary temperature, 250 °C; helium gas flow, 0.5 ml min^{-1}; isolation width, 1 amu; scan rate, 1000 amu s^{-1} and vapourization temperature, 250 °C. The same for MS/TOF studies were: hexapole Rf, 120.0 Vpp; collision cell Rf, 120.0 Vpp; pre-pulse storage, 3.0 μs; collision energy, 15 eV; quadrupole ion energy, 3.0 eV; nebulizer gas pressure, 1.2 bar; dry gas flow rate, 6.0 l min^{-1} and dry temperature, 180 °C. The drug was directly infused using a syringe pump at a concentration of 5 $\mu g\,ml^{-1}$ in MeOH:H_2O (50:50, v/v) at a flow rate of 180 $\mu l\,ml^{-1}$.

2.7. LC–MS/TOF studies on the degradation products

The mass studies on degradation products were carried out using LC–MS/TOF, employing the same gradient LC method and the mass parameters, as used for the drug.

3. Results and discussion

3.1. Optimization of the LC method

The analysis of individual reaction solutions by initial LC method and resultant chromatograms revealed overlap of polar degradation products appearing on the left of the drug. So the same was optimized by taking a mixture of reaction solutions (in which decomposition was observed) and changing various method parameters one by one. Acceptable separation was achieved using a mobile phase composed of MeOH (A): ammonium acetate buffer (B) (0.01 M, pH 4), employing the following gradient: T_{min}/A:B (v/v); $T_{0.01-10}$/1:99 (v/v), T_{15-20}/25:75 (v/v) and T_{25-30}/1:99 (v/v). The detection was carried out at 265 nm, temperature of the study was 25 °C, and flow rate was 1 ml min^{-1}.

3.2. Degradation behavior

The optimized LC method was used to study the degradation behavior of the drug under various stress conditions. The corresponding chromatograms are shown in Fig. 2. Unreacted drug solutions showed only one major peak, as depicted in a typical chromatogram in Fig. 2a.

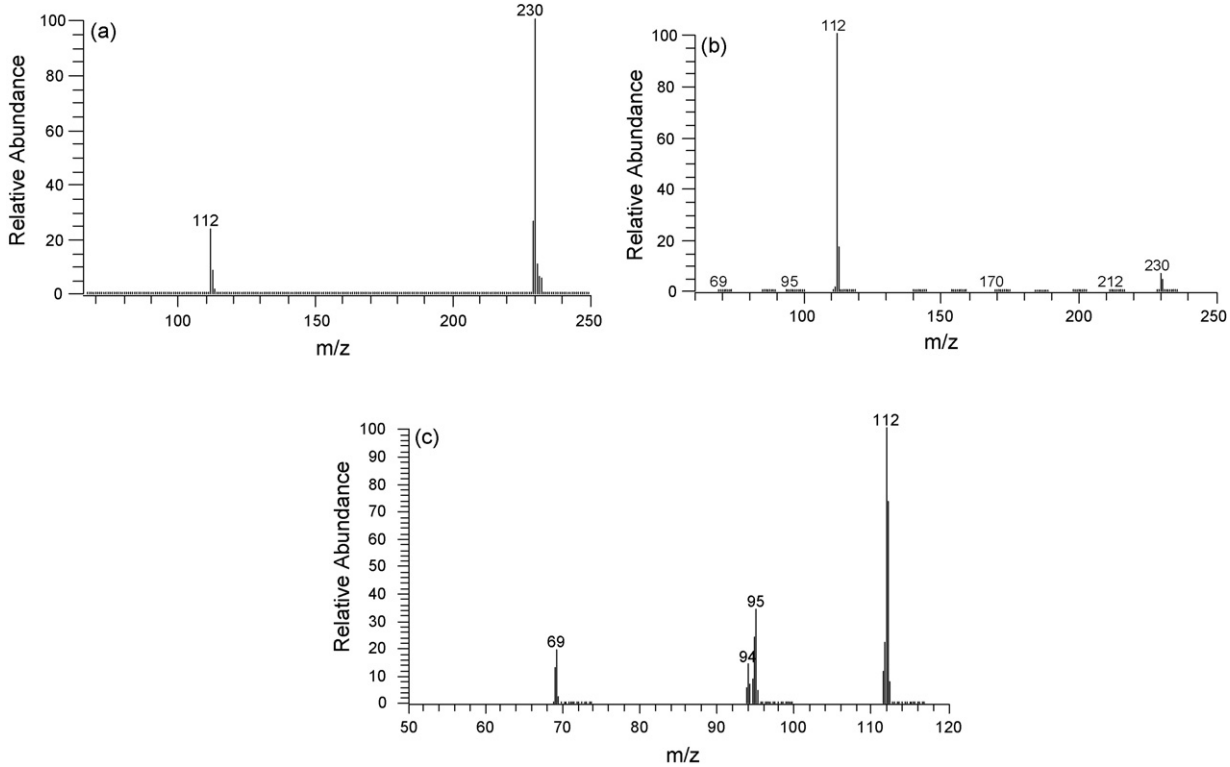


Fig. 3. Positive ion ESI mass spectra of lamivudine (MW = 230): (a) full scan spectra of $[M+H]^+$ ions of lamivudine, (b) product ion spectra (MS^2) of the ions of *m/z* 230 and (c) product ion spectra (MS^3) of the ions of *m/z* 112.

3.2.1. Hydrolysis

No additional peaks were generated on heating the drug in water for 72 h at 80 °C (Fig. 2b), indicating it to be stable under neutral condition. In comparison, three degradation product peaks, I, II and V were seen on treatment of the drug in 0.1N HCl for 48 h at 80 °C (Fig. 2c). The same products were produced even in 0.1N NaOH, where heating at 80 °C for smaller time period of 12 h resulted in higher extent of drug degradation (Fig. 2d).

3.2.2. Oxidative stress

There was insignificant degradation in 3% H_2O_2 after 48 h at room temperature. Instead, 35% decomposition occurred in 30% H_2O_2 after 48 h, resulting in products III and IV (Fig. 2e). The drug was lost completely into the same products on continued exposure to 30% peroxide for 72 h (Fig. 2f).

3.2.3. Photolysis

The drug proved to be stable to light. Almost nil to minimal decomposition was observed on exposure of acid and alkali drug solutions, and solid drug to light (Fig. 2g–i). The formation of small quantities of degradation products I and V after 10 days in alkali (Fig. 2h) was probably due to mild influence of base at low temperature of 40 °C, and not due to the light.

Table 4
Peak purity data of drug and degradation products.

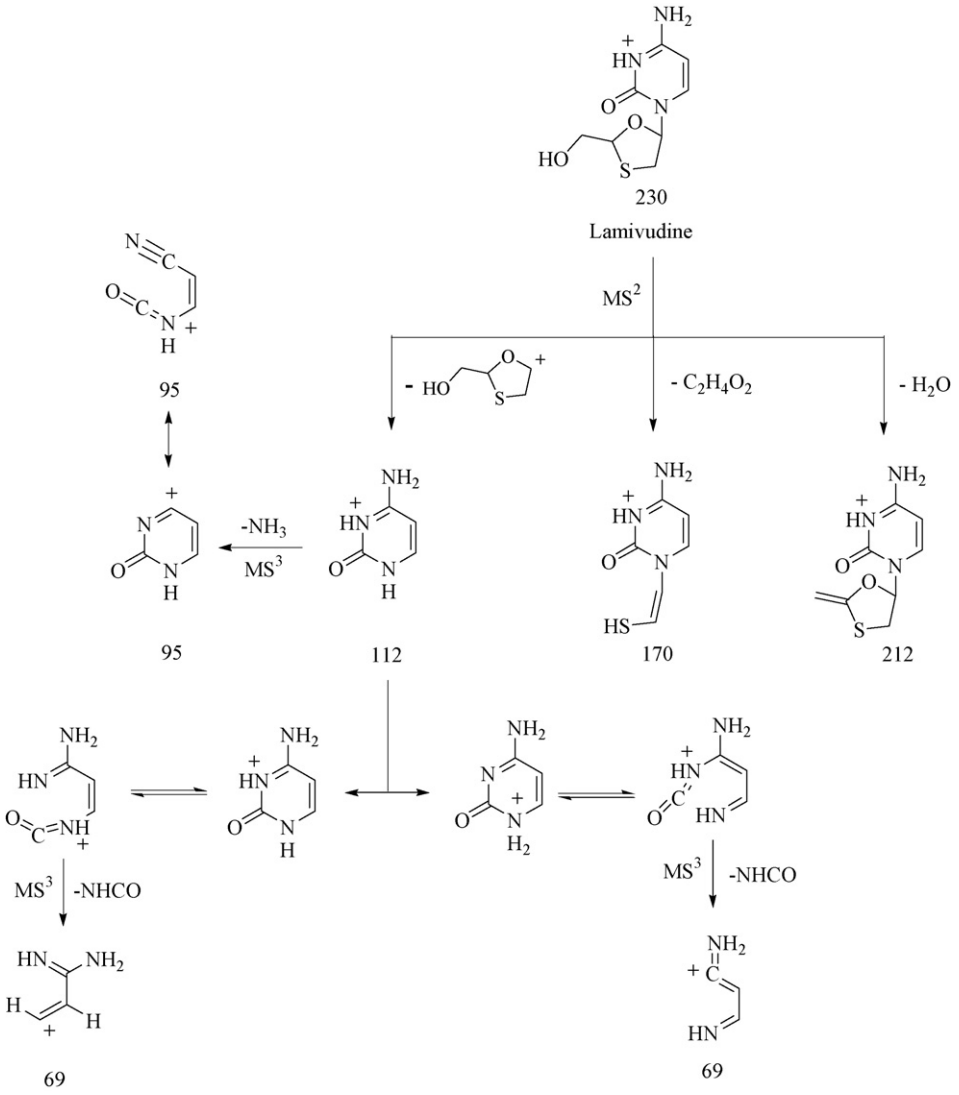
Drug/degradation products	Peak purity index	Peak purity threshold	Resolution factor
I	0.999600	0.989540	1.77
II	0.998054	0.955696	2.56
III	0.999987	0.999619	8.21
IV	0.999968	0.999762	3.46
Lamivudine	0.999985	0.999655	42.60
V	0.999581	0.994658	7.43

3.2.4. Solid state studies

The drug was stable to thermal stress in solid state. As shown in Fig. 2j, no change was observed on its exposure to dry heat at 50 °C for 2 months.

3.3. Validation of the LC method

The linearity of the method was established between concentration range of 50 μg ml^{-1} and 500 μg ml^{-1} (r^2 = 0.9997). The data for triplicate studies (Table 1) showed that %R.S.D. for each investigated concentration was <0.15%. The %R.S.D. for intra- and inter-day precision studies at three different concentrations, *viz.*,



Scheme 1. Fragmentation pathway of lamivudine based on MS^n study.

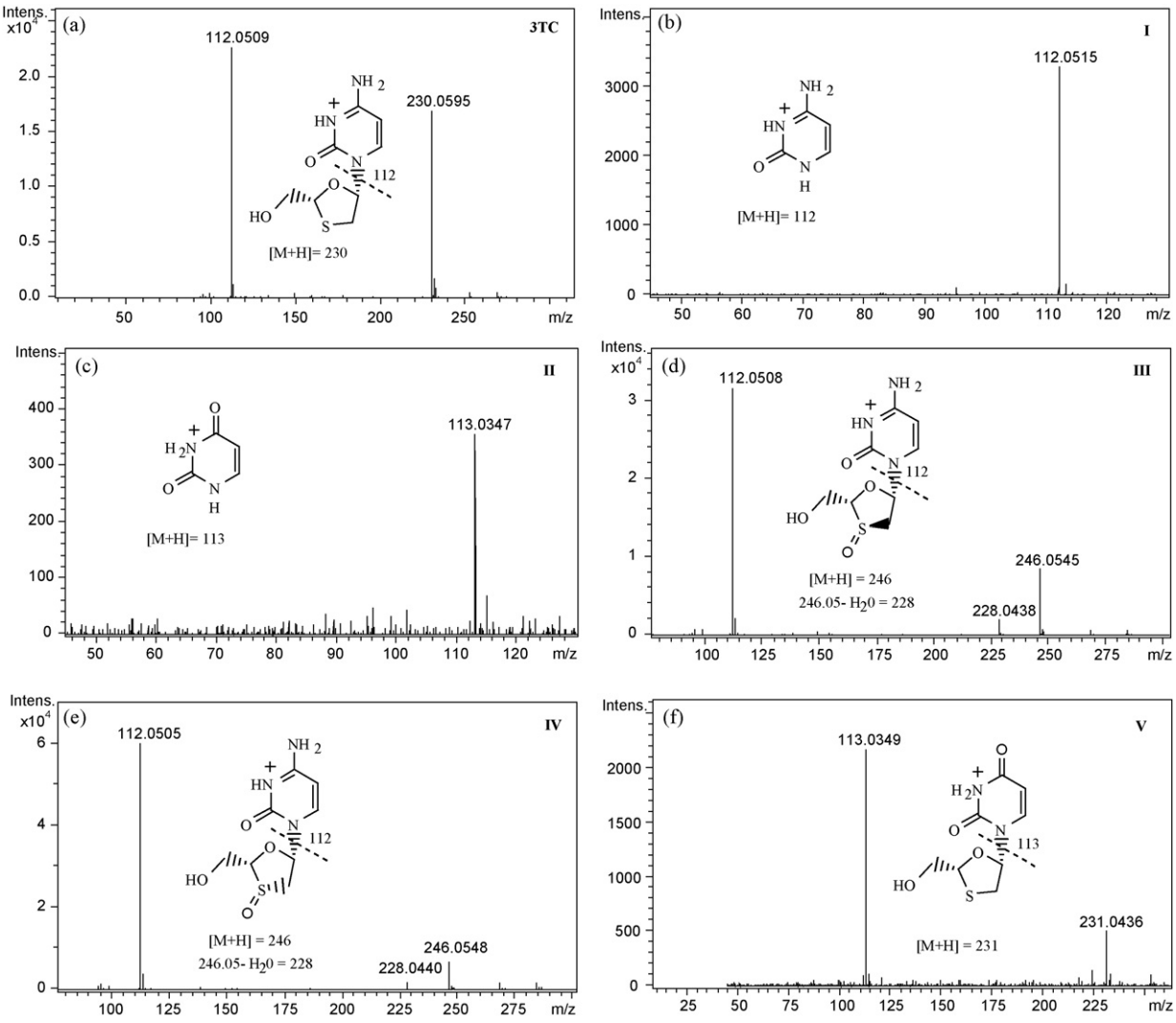


Fig. 4. Fragmentation pattern and mass spectra of degradation products of lamivudine.

100 μg ml^{-1}, 300 μg ml^{-1} and 500 μg ml^{-1} (Table 2) was <0.2%, indicating that the method was sufficiently precise. Also, good recoveries were obtained when a mixture of stressed samples was spiked with the drug at the above given three concentrations (mean recovery = 100.32%, Table 3). The method proved to be specific to each peak, which was indicated through peak purity data obtained using a PDA detector and also through resolution factor data, whose values were >1.5 for each peak (Table 4).

3.4. MS^n study and fragmentation pathway of the drug

The mass spectra of the drug (Fig. 3a) showed two peaks, one for molecular ion of *m*/*z* 230 and a fragment of *m*/*z* 112. The MS^2 analysis of the molecular ion of *m*/*z* 230 resulted in major product ion of *m*/*z* 112, and fragments of *m*/*z* 212, 170, 95 and 69 with relatively low abundance (Fig. 3b). Further, MS^3 studies revealed generation of fragments ions of *m*/*z* 69, 94 and 95 from major ion of *m*/*z* 112 (Fig. 3c).

The fragmentation pathway of the drug, based on the above-discussed MS^n data, is summarized in Scheme 1. It is proposed that the drug was ionized to protonated cytosine (*m*/*z* 112) on replacement of oxathiol moiety by hydrogen [16]. The subsequent generation of fragments of *m*/*z* 94, 95 and 69 from protonated cytosine during MS^3 study resulted from loss of H_2O, NH_3 and HNCO, respectively. As shown in Scheme 1, removal of NH_3 yielded two ions in equilibrium, wherein ring-opened structure could be considered thermodynamically more stable, based on the known

Table 5
Product code, relative retention time, molecular formula, calculated mass, observed accurate mass and error in ppm.

Product	Relative retention time	Theoretical mass for $[M+H]^+$ ions	Observed mass for $[M+H]^+$ ions	Error (ppm)	Molecular formula
I	0.15	112.0511	112.0515	3.6	$C_4H_5N_3O$
II	0.22	113.0351	113.0347	−3.5	$C_4H_4N_2O_2$
III	0.32	246.0549	246.0545	−1.6	$C_8H_{11}N_3O_4S$
IV	0.38	246.0549	246.0548	−0.4	$C_8H_{11}N_3O_4S$
Lamivudine	1.00	230.0599	230.0595	−1.7	$C_8H_{11}N_3O_3S$
V	1.10	231.0440	231.0436	−1.7	$C_8H_{10}N_2O_4S$

behavior in the literature [16]. The ion of *m*/*z* 94 resulted from neutral loss of water from cytosine, which is also reported previously [17]. There were two possible structures for the ion of *m*/*z* 69, as shown in the scheme. This was due to probability of protonation of cytosine at different nitrogens in the ring of the molecule. In comparison to this complex cytosine-mediated fragmentation pathway, the formation of fragments with *m*/*z* of 170 and 212 was rather simple. The fragment of *m*/*z* 170, containing nucleobase and a part of sugar, resulted from rupture of sugar under the influence of sulfur atom [18]. This was because, unlike other nucleosides, lamivudine does not undergo retro Diels–Alder-mediated fragmentation, due to the presence of thiol group in the sugar moiety. The ion of *m*/*z* 212 resulted from loss of water from the drug.

3.5. LC–MS/TOF studies on drug and mixture of forced degraded samples

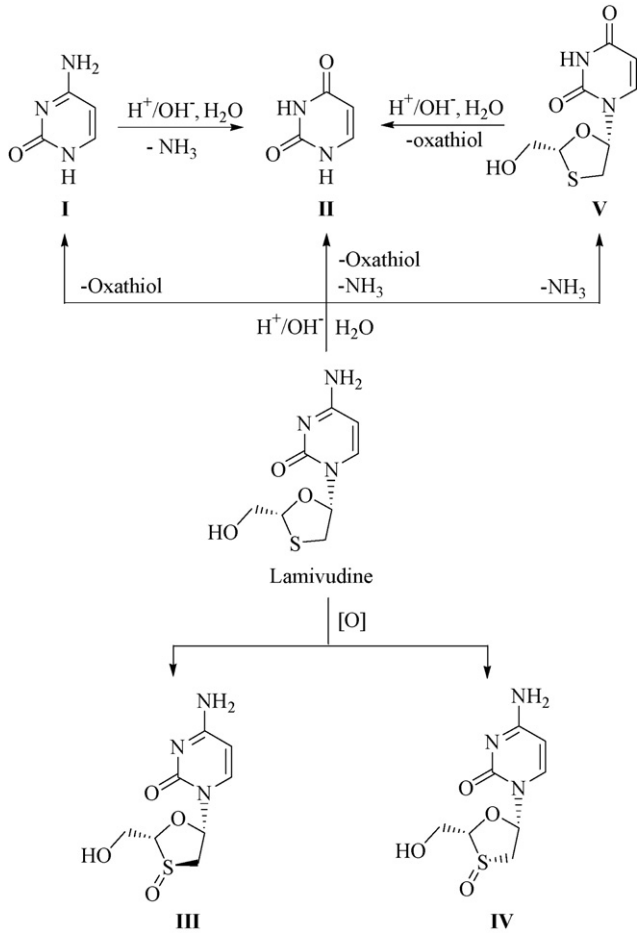
The drug and mixture of stressed samples were subjected to LC–MS/TOF studies using the optimized chromatographic conditions. The MS/TOF spectra of drug and degradation products are shown in Fig. 4. Table 5 lists the theoretical and observed accurate mass values for drug and degradation products, along with error in ppm and determined molecular formula of each. As shown, the error in ppm between theoretical and observed accurate masses was within acceptable limits in each case, thus justifying the obtained chemical formulae.

3.6. Postulated structures of degradation products

As shown in Fig. 2, products I and II resolved earliest, indicating their higher polarity in comparison to the drug. Also, the protonated molecular ion peaks of the two had small mass, *viz*., *m*/*z* 112 (Fig. 4b) and *m*/*z* 113 (Fig. 4c), respectively, indicating cleavage of the molecule. This was considered to happen at the glycosidic bond, based on the known behavior of nucleosides [19]. The same was supported even by their elemental composition, calculated from accurate masses, as $C_4H_5N_3O$ and $C_4H_4N_2O_2$, respectively (Table 5). The absence of sulfur in the molecular formulae clearly indicated release of the oxathiol moiety in both. While the structure of I was established to be 4-aminopyrimidin-2(1*H*)-one (cytosine), the mass and molecular formula of II indicated removal of NH_3 group and addition of water, so it was convincingly considered to be pyrimidine-2,4(1*H*,3*H*)-dione (uracil). The structure of II was supported by the fact that nucleic acids are easily deaminated under acidic and alkaline conditions [19].

V, the third major degradation product in acidic and alkaline condition, resolved beyond but close to the drug (Figs. 2c and d), indicating it to be drug-related relatively non-polar compound. Its mass spectrum showed protonated molecular ion peak of *m*/*z* 231, with a single major fragment of *m*/*z* 113. Comparison of its molecular formula and fragmentation pattern with the drug (Fig. 4a versus f) indicated the same parallelism as among the degradation products I and II (Fig. 4b and c), which was true even with respect to relative retention behavior of the two sets. The whole pattern and data pointed towards deamination of the drug and subsequent water attack at the nucleobase moiety, similar to relationship between I and II. Based on the behavior, the structure was characterized as 1-[(2*R*,5*S*)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]pyrimidine-2,4(1*H*,3*H*)-dione.

The two oxidative degradation products, III and IV, yielded entirely similar LC–MS/TOF spectra (Fig. 4d and e), with protonated molecular mass of *m*/*z* 246 and fragments of *m*/*z* 228 and *m*/*z* 112. The accurate mass measurements suggested elemental composition of both as $C_8H_{11}N_3O_4S$. Based on the behavior, the two were concluded as diastereomers of each other. The fragment of *m*/*z* 228 could be attributed to loss of water and that of *m*/*z* 112 indicated changes in oxathiol part of the drug. The oxidation of sulfur in the oxathiol moiety was indicated from the difference of 16 amu between mass of the drug and the oxidation products. On this basis, the structures of the two were worked out to be 4-amino-1-[(2*R*,3*S*,5*S*)-2-(hydroxymethyl)-3-oxo-1,3λ4-oxathiolan-5-yl]pyrimidin-2(1*H*)-one and 4-amino-1-[(2*R*,3*R*,5*S*)-2-(hydroxymethyl)-3-oxo-1,3λ4-oxathiolan-5-yl]pyrimidin-2(1*H*)-one.



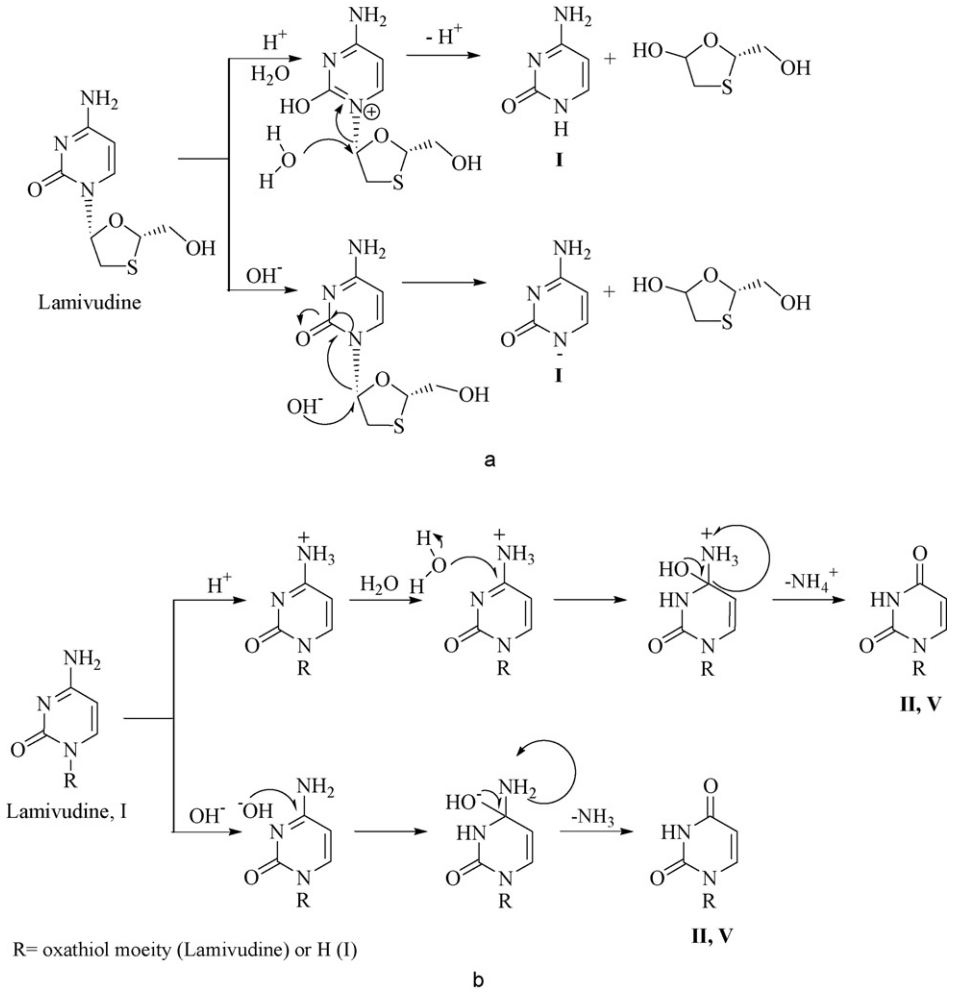
Scheme 2. Degradation pathway of lamivudine.

The structures for I–V were found to match exactly to impurities E–H, J, respectively, in the WHO monograph on the drug [11]. This further substantiated the proposed structures, and also helped to identify the degradation products among the impurities listed in the monograph.

3.7. Degradation pathway and mechanisms

The elucidation of structures of degradation products revealed three susceptible sites on the drug, *viz*., N-glycosidic bond, amino group in the nucleobase, and sulfur in the oxathiol ring. The amino group and glycosidic bonds showed susceptibility to hydrolytic cleavage, while sulfur in the oxathiol ring, as expected, was vulnerable to oxidation. Based on this, the projected degradation pathway of the drug leading to products I–V is proposed in Scheme 2.

The mechanism of the formation of product I in acid and alkali conditions is described in Scheme 3a, which is based on the reported hydrolytic behavior of drugs with a glycosidic bond in



Scheme 3. Postulated mechanisms for the decomposition of lamivudine to products I, II and V in the presence of acid and alkali.

the molecule [19,20]. The same mechanism even explains the formation of product II (Scheme 3b) from the drug, and even from product V (Scheme 2). Products II and V are proposed to be generated in acid conditions form drug or I through spontaneous attack of water on the protonated species, or through direct attack of hydroxyl ion in alkaline medium, followed by removal of ammonia (Scheme 3b).

The oxidative diastereomeric products III and IV are postulated to form by the direct attack of oxygen on the sulfur moiety of oxathiol ring. The mechanism is supported by the literature [21].

4. Conclusion

The study was able to yield the following new and useful information, yet not reported in the literature on lamivudine: (i) a validated stability-indicating LC method, suitable for extension to LC–MS studies, (ii) sensitivity of drug to various stress degradation conditions, (iii) total number and the nature of degradation products formed under different stress conditions, (iv) pathways of mass fragmentation of the drug and degradation products, (v) pathway of decomposition of the drug, (vi) mechanistic explanation to the origin of degradation products and (vii) identification of degradation products among the list of impurities in the WHO monograph.

Acknowledgement

The support provided by M/s Thermo Fischer Scientific (Mumbai, India) in carrying out MS^n studies is duly acknowledged.

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