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LC–MS/MS studies of ritonavir and its forced degradation products

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ABSTRACT

Forced degradation of ritonavir (RTV), under the conditions of hydrolysis (acidic, basic and neutral), oxidation, photolysis and thermal stress as prescribed by ICH was studied using LC–MS/MS. Eight degradation products were formed and their separation was accomplished on Waters XTerra® C_{18} column (250 mm × 4.6 mm i.d., 5 μm) using water:methanol:acetonitrile as (40:20:40, v/v/v) mobile phase in an isocratic elution mode by LC. The method was extended to LC–MS/MS for characterization of the degradation products and the pathways of decomposition were proposed. No previous reports were found in the literature regarding the characterization of degradation products of ritonavir.

1. Introduction

Protease inhibitors are responsible for marked reductions in morbidity and mortality in patients with advanced human immunodeficiency virus (HIV) infection [1]. Inhibition of HIV protease is one of the most important approaches for therapeutic intervention of acquired immunodeficiency syndrome (AIDS). Ritonavir (RTV) is a selective, competitive and reversible inhibitor of both HIV-1 and HIV-2 proteases. It is widely used in the treatment against AIDS and particularly to inhibit liver enzyme, viz., cytochrome P450-3A4 (CYP3A) [2]. It is not only used on its own as an antiretroviral but also a booster of other anti-HIV drugs to slow and/or prevent the onset of AIDS. Chemically, RTV is (5S,8S,10S,11S)-10-hydroxy-2-methyl-5-(1-methylethyl)-1-[2-(1-methylethyl)-4-thiazolyl]-3,6-dioxo-8,11-bis(phenylmethyl)-2,4,7,12-tetraazatridecan-13-oicacid,5-thiazolylmethyl ester (Fig. 1).

Several HPLC methods were published for determination of antiretrovirals including RTV in different formulations [1–4]. HPTLC was used for simultaneous determination of ritonavir and lopinavir in capsules [5]. A few CE and LC–MS methods were reported for analysis of RTV and its metabolites in biological fluids [6–9]. International pharmacopoeia (Ph.Int.) describes a LC method to separate RTV and its impurities. However, no brand names of columns (L1) were mentioned in pharmacopoeial texts, due to which analysts often face difficulty in selecting a suitable stationary phase. Recently, Yekkala et al. [2], have evaluated the pharmacopoeia method to check the suitability of a set of commercial columns and found that Hypersil BDS complies with system suitability requirement of Ph.Int. However, studies on degradation behavior of RTV were limited in the literature. A stability indicating assay of RTV in soft gelatin capsules was reported by Dias et al. [1]. In another report, Donato et al. [3], studied the forced degradation of ritonavir and lopinavir under hydrolytic (acid, base and neutral), oxidative and photolysis. However, in both cases, neither the characterization of degradation products nor the degradation pathways were reported. The International Conference on Harmonization (ICH) guidelines [10], suggest stress studies on a drug to establish its inherent stability characteristics not only for identification of degradation products but also understanding the stability of drug molecule. So, it is of great importance to know the complete degradation profile of RTV, which is not yet reported in the literature.

The present manuscript describes the degradation behavior of RTV under hydrolysis (acidic, basic and neutral), oxidation, thermal and photolysis conditions. Optimization of LC conditions to separate the drug and its degradation products on a reverse phase C_{18} column, method validation, characterization of degradation products, and mechanism of degradation by LC–MS/MS were discussed.

2. Experimental

2.1. Chemicals and reagents

Analytical grade reagents and HPLC grade solvents were used. Glass-distilled and deionized water NANOpure (Branstead, USA)

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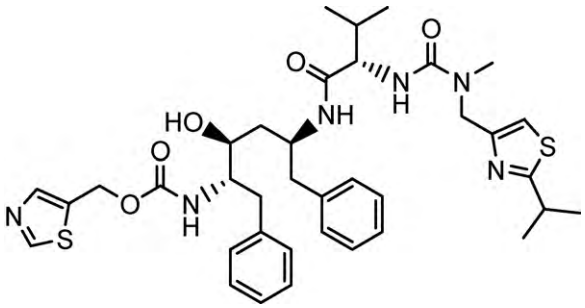

Fig. 1. Chemical structure of ritonavir (RTV).

was used. Methanol and acetonitrile were purchased from Qualigens Fine Chem (Mumbai, India). Sodium hydroxide, hydrochloric acid, and hydrogen peroxide purchased from S.D. Fine Chem Ltd. (Mumbai, India). Ritonavir API was a gift sample from a local manufacturing unit in Hyderabad.

2.2. Instrumentation

An HPLC system consisting of two LC-20AD pumps, SPD-M20A diode array detector, SIL-20AC auto sampler, DGU-20A_3 degasser, and CBM-20A system controller (all from Shimadzu, Kyoto, Japan) were used. A reverse phase Waters XTerra® C_{18} column (250 mm × 4.6 mm i.d., 5 μm) was used for separation of all the compounds. The chromatographic data were recorded using an HP-Vectra (Hewlett Packed, Waldron, Germany) computer system with LCsolution data acquiring software (Shimadzu, Kyoto, Japan). LC–MS/MS was performed by Agilent 1100 series online ion trap MSD mass spectrometer with APCI source in positive mode equipped with an auto sampler (G1329A), and diode array detector (G1315B) (All from Agilent technologies, Waldbronn, Germany). The data was acquired and processed using LC/MSD trap software 4.2 (Bruker, Waldbronn, Germany). The high resolution mass spectrometry (HRMS) data was 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.

2.3. Forced degradation

Stress studies were carried out according to ICH guidelines Q1A(R_2) [10]. For each stress condition, the sample was dissolved in 6.0 mL of methanol and remained in solution when acid, caustic, hydrogen peroxide, or water was added to obtain a concentration of 25 mg/25 mL. The API was subjected to forced degradation under acidic, basic and neutral conditions by refluxing in 0.5 N HCl at 75 °C, 0.1N NaOH at room temperature and distilled water at 75 °C for 12, 72 and 30 h, respectively. Oxidative stress was carried out using 30% H_2O_2 at room temperature. The drug was placed in a thermally controlled oven at 75 °C up to 6 days for thermal stress in solid as well as solution forms. For photolytic stress, the drug was exposed to sunlight for 60 h in solid as well as solution forms.

2.4. Sample preparation

The stressed samples of acid and base hydrolysis were neutralized and diluted with mobile phase to obtain 100 μg/mL solutions. Neutral hydrolysis, thermal and photolytic samples were diluted with mobile phase to obtain100 μg/mL solutions. The oxidative stress sample was diluted with mobile phase to obtain 10 μg/mL solution. All the prepared samples were passed through 0.45 μm membrane filter before HPLC analysis.

2.5. Chromatographic and mass spectrometric conditions

The LC separation was carried out by Waters XTerra® C_{18} column (250 mm × 4.6 mm i.d., 5 μm) using water:methanol:acetonitrile (40:20:40, v/v/v) as a mobile phase in an isocratic mode and detection by photo diode array detector at 210 nm at 25 °C. The flow rate was 1.0 mL/min and injection volume 10 μL. LC–MS/MS was carried out by atmospheric pressure chemical ionization (APCI), in positive ion mode of detection. Nitrogen was used as a nebulizer gas. Collision-induced dissociation was achieved by helium as a collision gas. The operating conditions for MS scan were: temperature, 325 °C; nebulizer gas, 60 psi; dry gas, 5.0 mL/min; fragmentation amplifier, 0.50 V; current corona, 4000 nA; skimmer, 40.0 V; capillary exit, 138.6 V; vaporizer temperature, 400 °C and dwell time, 300 ms. The HRMS data was 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 quadrupole analyzer and TOF analyzer analyzed the product ions.

2.6. Validation parameters

A stock solution containing 5 mg/5 mL drug was diluted to yield solutions in the concentration range of 10–200 μg/mL to establish linearity and range. The analysis was carried out in triplicate by injecting 10 μL of each solution. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. The intra- and inter-day precision were established by analyzing 50, 100 and 200 μg/mL of drug solution, three times on the same day and on three consecutive days (Table 1), respectively. Accuracy was determined by analyzing a known concentration of drug, viz., 20, 50 and 100 μg/mL spiked with stressed sample in triplicate and then determining the percent recovery (Table 1). The signal to noise ratios were 3:1 and 10:1 for determining LOD and LOQ, respectively.

3. Results and discussion

3.1. Optimization of chromatographic conditions

During the optimization cycle, several conditions were tried on Waters XTerra® C_{18} column (250 mm × 4.6 mm i.d., 5 μm). Various mobile phases like methanol and water, acetonitrile and water in different proportions were tried in an isocratic mode. To detect the drug and degradants with sufficient peak intensity, the wavelength 210 nm was selected. It was found that, at least 60% of organic modifier was needed to elute all peaks within 10 min due to high lipophilicity. Neither methanol nor acetonitrile gave sufficient resolution alone. Mixtures of water: methanol: acetonitrile in different proportions were used. During the development cycle, a mobile phase consisting of water:methanol:acetonitrile (40:20:40, v/v/v) at a flow rate of 1.0 mL/min and PDA detection at 210 nm, in an isocratic mode gave good separation of the drug and its degradation products. The advantage of the method was simple and rapid. The optimized LC conditions were extended to LC–MS/MS studies.

3.2. Validation

The method was validated and good linearity was found in the concentration range 10–200 μg/mL (r^2 = 0.9992) of the drug. The data for triplicate analysis showed that the % R.S.D. for each investigated concentration was <0.25%. The % R.S.D. for intra- and

Table 1
Precision and recovery data of RTV.

Precision data			
Conc., μg/mL	50	100	200
Intra-day precision			
Measured conc., (μg/mL), ±S.D., R.S.D. (%)	50.00 ± 0.11, 0.222	100.00 ± 0.25, 0.250	199.64 ± 0.27, 0.314
Inter-day precision			
Measured conc., (μg/mL), ±S.D., R.S.D. (%)	50.00 ± 0.19, 0.375	100.00 ± 0.21, 0.231	199.99 ± 0.26, 0.129
Recovery data			
Conc., μg/mL	20	50	100
Calculated spiked conc., (μg/mL), ±S.D., R.S.D. (%)	20.92 ± 0.01, 0.021	50.81 ± 0.17, 0.342	98.32 ± 0.15, 0.152
Recovery (%) $n = 7$	101.58	101.61	98.32

n = no. of replicates.

Table 2
Retention data of RTV and its degradation products.

Degradation product	Code	m/z	Retention time (R_t) min	Capacity factor (k^1)	Resolution factor (R_s)	Asymmetry factor (A_s)
–	RTV	721.3	9.0	3.46	19.39	1.10
A1	–	ND	2.6	0.30	2.84	1.26
A2	DP1	582.2	4.7	1.37	13.87	1.21
A3	DP2	707.3	7.2	2.61	8.37	1.12
B1	–	ND	2.6	0.30	3.13	1.31
B2	DP3	116.0	2.9	0.47	2.43	1.34
B3	DP4	622.3	5.3	1.64	9.83	1.19
B4	DP5	436.2	6.3	2.15	3.61	1.13
B5	DP6	171.0	8.0	2.96	5.40	1.05
B6	DP7	606.3	9.2	3.57	8.08	1.08
N1	DP3	116.0	2.9	0.47	4.75	1.30
N2	DP4	622.3	5.3	1.64	9.83	1.13
N3	DP5	436.2	6.3	2.15	3.61	1.14
N4	DP6	171.0	8.0	2.96	5.47	1.02
N5	DP7	606.3	9.2	3.57	8.08	1.09
O1	DP8	753.3	5.9	1.93	2.64	1.32
T1	DP3	116.0	2.9	0.47	4.75	1.30
T2	DP4	622.3	5.3	1.64	9.83	1.10
T3	DP5	436.2	6.3	2.15	3.55	1.14
T4	DP6	171.0	8.0	2.96	5.45	1.01
T5	DP7	606.3	9.2	3.57	8.08	1.09
P1	DP1	582.2	4.7	1.37	16.24	1.07

inter-day precision at three different concentrations, viz., 50, 100, and 200 μg/mL was <0.40% (Table 1). Also, good recoveries were obtained when stressed samples were spiked with known concentration of the drug at 20, 50, and 100 μg/mL with mean recovery 100.50% (Table 1). The limits of detection (LOD) and quantification (LOQ) were found to be 10 and 30 ng/mL, respectively. The capacity (k^1) and resolution factors (R_s) were calculated and the chromatographic parameters are given in Table 2. It could be seen from Table 2 that all the peaks were well resolved.

4. Degradation of RTV

The degradation behavior of RTV under various stress conditions was investigated by LC. Typical chromatograms are shown in Fig. 2.

4.1. Hydrolysis

Initially RTV was refluxed in 1.0N HCl at 75 °C for 24 h. Complete degradation of the drug was observed. When the strength of the acid was reduced to 0.5N HCl, 40% degradation was observed in 12 h. Three degradation products (A1–A3) were formed on acid hydrolysis (Fig. 2b), which were coded as A2-DP1 and A3-DP2. Six degradation products (B1–B6) on treatment of the drug with 0.1N NaOH for 72 h at room temperature were formed. The codes provided for B2–B6 were DP3–DP7, respectively. Under neutral hydrolysis, five degradation products were formed after 30 h at 75 °C. These products could be represented as DP3–DP7 for N1–N5, respectively. The peaks A1 and B1 were not coded because they were not detected by LC–MS.

4.2. Oxidative stress

Oxidative stress was carried out using 30% H_2O_2 at room temperature. Only one degradation product O1 (DP8) was formed after 48 h. A small peak at 5.9 min was eluted (Fig. 2e).

4.3. Thermal stress

The drug in solid form was kept at 75 °C for 6 days. No significant degradation was observed. However, the drug in solution form formed five degradants (T1–T5). Similarly, the peaks from T1 to T5 could be represented as DP3–DP7, respectively. The degradants formed under neutral and thermal hydrolysis were overlapped. However, the rate of degradation was high under neutral hydrolysis (kept under reflux condition) when compared to thermal hydrolysis (kept in a thermally controlled oven) (Fig. 2d and f).

4.4. Photolysis

The solid form of the drug remained stable on exposure to sunlight for 60 h. But only one degradation product P1 (DP1) was

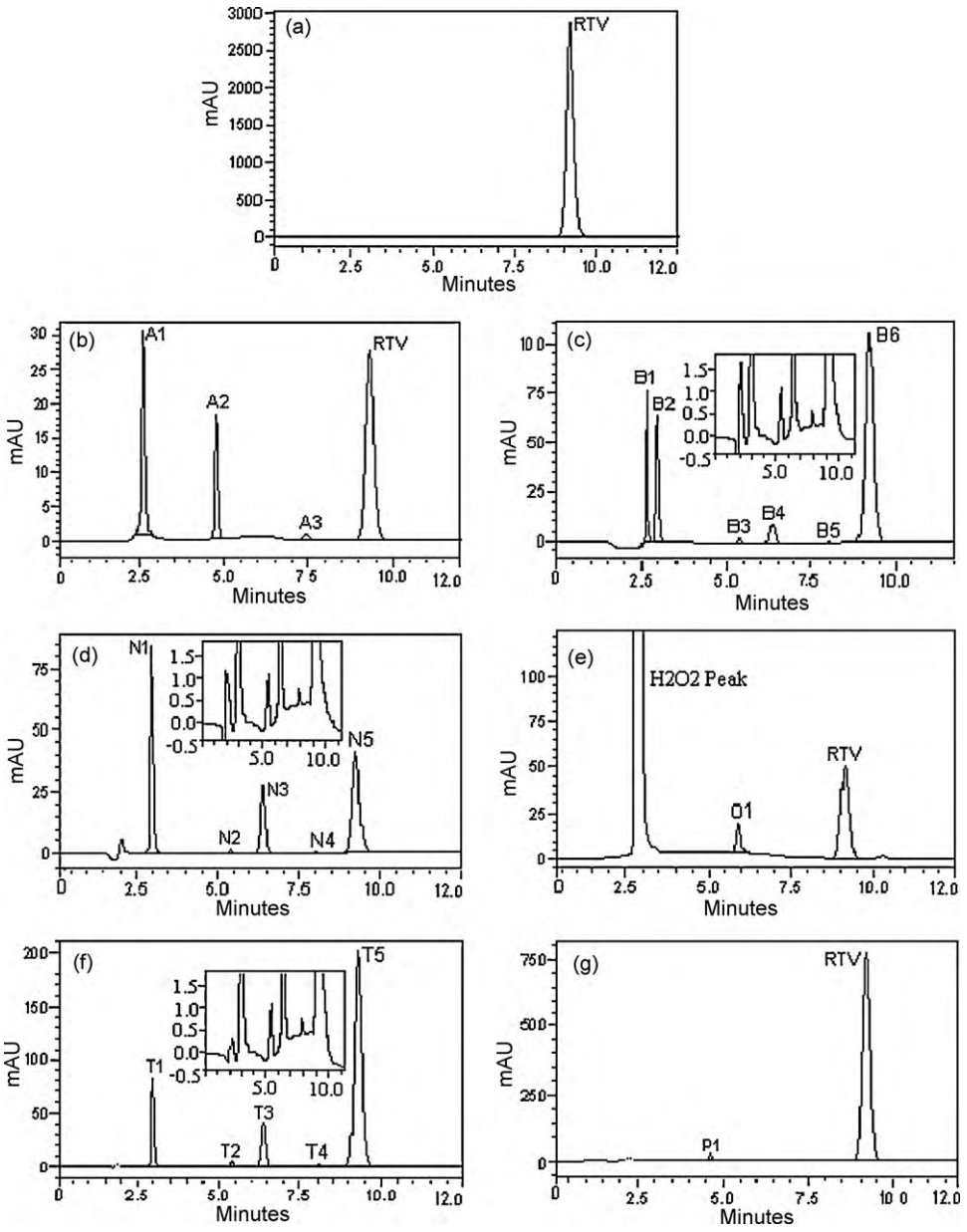

Fig. 2. Typical HPLC chromatograms of (a) undegraded API (RTV) and its degradation products formed under (b) acidic, (c) basic, (d) neutral, (e) peroxide, (f) thermal and (g) photolytic conditions.

obtained in solution form after 60 h, which was eluted at the same retention time of degradant A2.

4.5. Mass spectral fragmentation

The analysis of the degradation products was carried by LC and LC–MS. RTV was subjected to LC–MS/MS with atmospheric pressure chemical ionization (APCI) to know the fragmentation pattern of drug. The MS^2 analysis of the precursor ion (m/z 721) of the drug gave four product ions at m/z 426, 296, 268 and 171. These ions were represented as A–D, respectively (Fig. 3). Table 3 lists the observed and theoretical accurate mass values for drug and fragment ions, along with error in ppm and determined molecular formula of each. The ions at m/z 426 and 296 were generated through the cleavage of the amide bond [11]. The fragment ion at m/z 296 was relevant to oxazalone moiety. Nevertheless, similar fragmentation pattern was observed in peptides and proteins [12]. The fragment at m/z 268 was formed by the cleavage of keto-isopropyl linkage. The fourth fragment ion at m/z 171 was formed through cleavage of C–N bond in urea derivative. The fragmentation pathway of RTV is summarized in Fig. 3.

4.6. Characterization of degradation products

The degradation products were subjected to HRMS studies in ESI positive mode to determine their molecular ion peaks and to establish their fragment profile. Fig. 4 lists the m/z values of product ions and its fragment ions. 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 4. A difference of 139 amu between the fragment at m/z 582 and precursor ion m/z 721 suggested that DP1 was formed by loss of the 2-isopropyl-4-methylthiazole ion in the form of (2-isopropylthiazol-4-yl)methanol (157 amu) during the hydrolysis

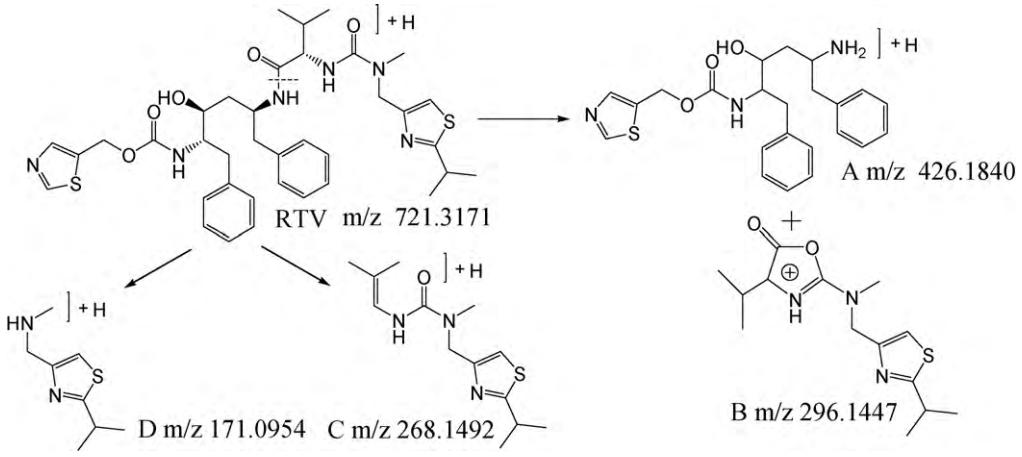

Fig. 3. Mass spectral fragmentation of ritonavir (RTV).

Table 3
HRMS data of M+H and other fragment ions of RTV.

Fragment ion	Observed mass (amu)	Best possible molecular formula	Theoretical mass (amu)	Error (ppm)	RDB
M+H	721.3171	$C_{37}H_{49}N_6O_5S_2$	721.3205	−4.83	16.5
A	426.1840	$C_{23}H_{28}N_3O_3S$	426.1851	−2.67	11.5
B	296.1447	$C_{14}H_{22}N_3O_2S$	296.1432	4.81	5.5
C	268.1492	$C_{13}H_{22}N_3OS$	268.1483	3.13	4.5
D	171.0954	$C_8H_{15}N_2S$	171.0955	−1.14	2.5

RDB: rings plus double bonds.

[13,14]. The accurate *m*/*z* value of DP1 was 582.2831 Da. The same was supported even by their elemental composition, calculated from accurate masses, as $C_{30}H_{40}N_5O_5S^+$. The combination of fragment ions 426 and 157 resulted in DP1 (Fig. 5). The peak at 2.6 min was not detected by LC–MS, possibly due to poor ionizability, having an absorbance of 75 mAU (Fig. 2b). The 157 amu peak was also not detected by LC–MS. In case of DP2, the experimental *m*/*z* value was 707.3081 Da and its suggested elemental composition was $C_{36}H_{47}N_6O_5S_2^+$. A difference of 14 amu between the fragment ion at *m*/*z* 707 and precursor ion (*m*/*z* 721) could be attributed to loss of $-CH_2$ or nitrogen atom from drug molecule. However, the loss of nitrogen atom was ruled out according to nitrogen rule and presumed that one methylene group was eliminated in the form of CH_3OH during hydrolysis. The fragment ions at *m*/*z* 282

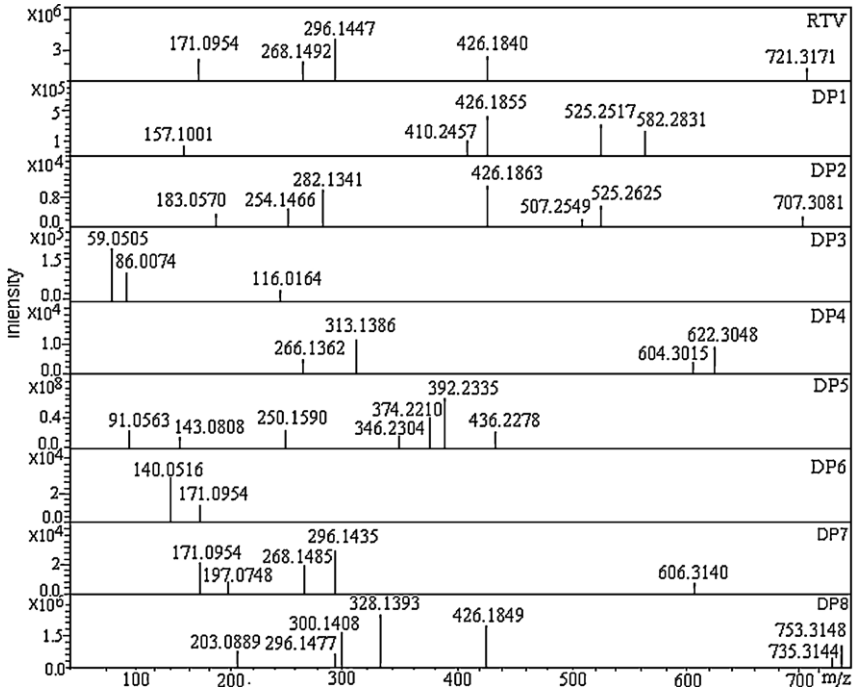

Fig. 4. Mass spectra of RTV and degradation products (DP1–DP8).

and 183 supported the absence of methyl group at urea moiety (Fig. 5). The molecular ion peak DP3 (m/z 116.0164 Da) formed by hydrolysis of the carbamate from drug leads to the formation of (thiazole-5-yl)methanol. This could be a primary degradant pair of m/z 606.3140 Da (DP6), which was eluted at 2.9 min having an absorbance of 65 mAU under base hydrolysis (Fig. 2c). The presence of fragments of m/z of 86 and 59 indicated that the thiazole ring was intact in the product (Figs. 5 and 6). The accurate mass obtained from HRMS for DP4 was 622.3048 Da. The possible elemental composition suggested by elemental composition calculator was $C_{33}H_{44}N_5O_5S^+$. Its structure was justified by the loss of 5-methylthiazole, followed by cyclization. There was no possibility, for loss of 99 amu from precursor ion other than carbamate site, must be converted to (thiazole-5-yl)methanol (DP3) during hydrolysis. The fragment ions 604 and 313 not only supported the proposed structure but also satisfied the nitrogen rule.

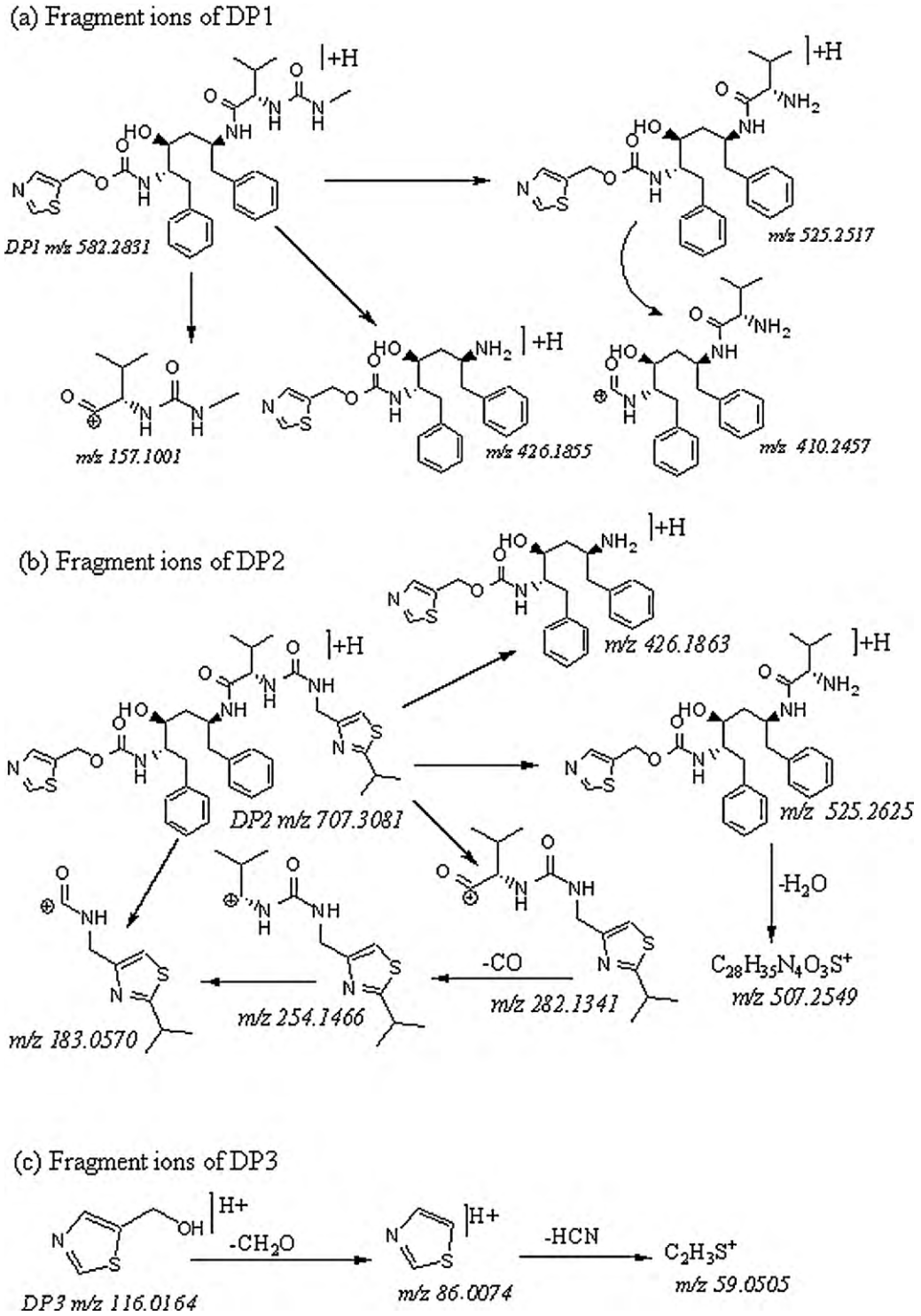

Fig. 5. Fragmentation pattern of degradation products: (a) DP1, (b) DP2, (c) DP3, (d) DP4, (e) DP5, (f) DP6, (g) DP7 and (h) DP8.

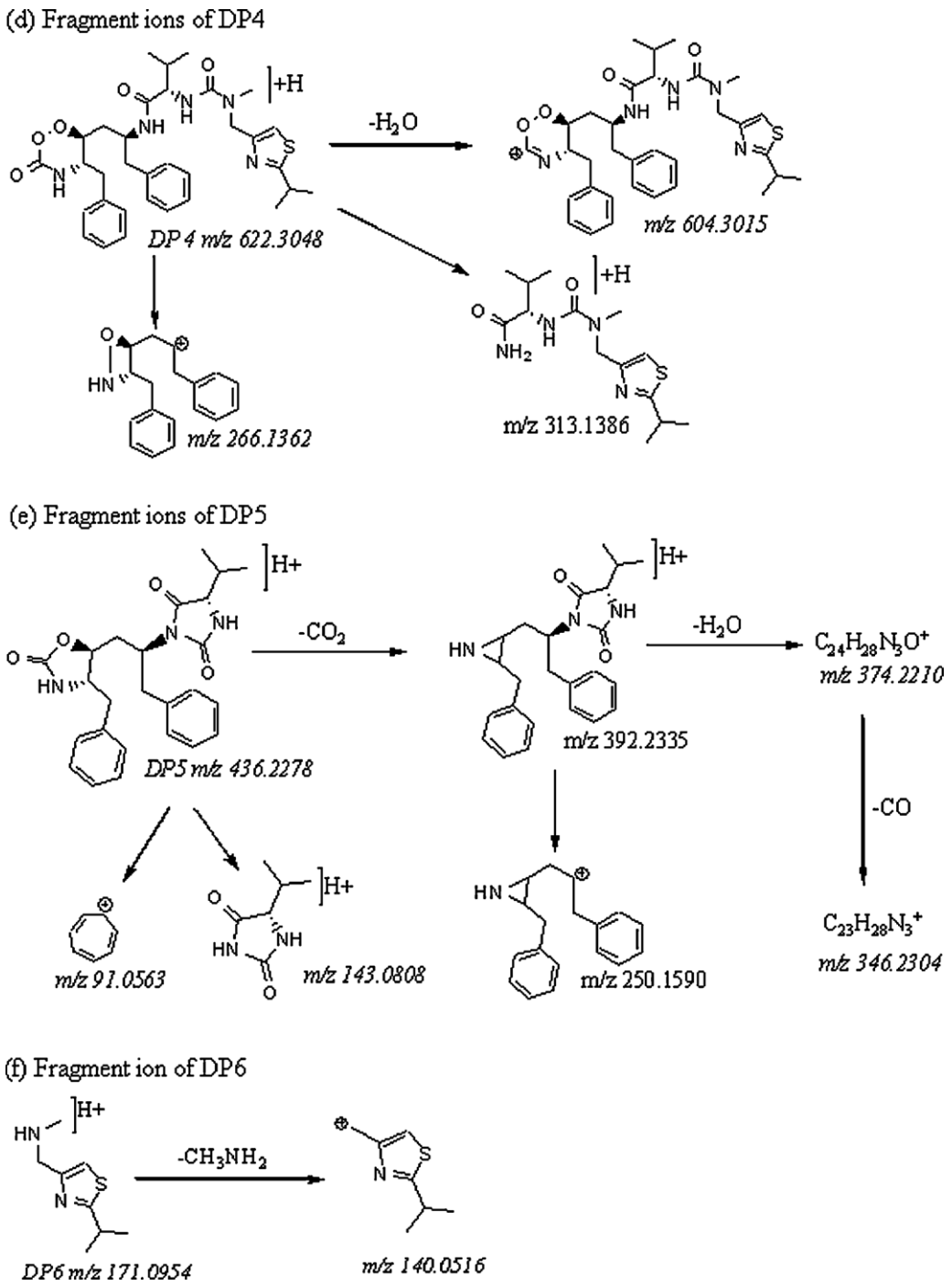

Fig. 5. (*Continued*)

Table 4
HRMS data of degradation products (DP1–DP8).

DP	Observed mass of $[M+H]^+$ ion (amu)	Probable molecular formula	Theoretical mass of $[M+H]^+$ ion (amu)	Error (ppm)	RDB
DP1	582.2831	$C_{30}H_{40}N_5O_5S^+$	582.2750	13.9	13.5
DP2	707.3081	$C_{36}H_{47}N_6O_5S_2^+$	707.3049	4.5	16.5
DP3	116.0164	$C_4H_6NOS^+$	116.0170	0.8	2.50
DP4	622.3048	$C_{33}H_{44}N_5O_5S^+$	622.3063	−7.7	14.5
DP5	436.2278	$C_{25}H_{30}N_3O_4^+$	436.2236	9.6	12.5
DP6	171.0954	$C_8H_{15}N_2S^+$	171.0955	−1.1	2.50
DP7	606.3140	$C_{33}H_{44}N_5O_4S^+$	606.3114	4.9	14.5
DP8	753.3148	$C_{37}H_{49}N_6O_7S_2^+$	753.3104	5.8	16.5

RDB: rings plus double bonds.

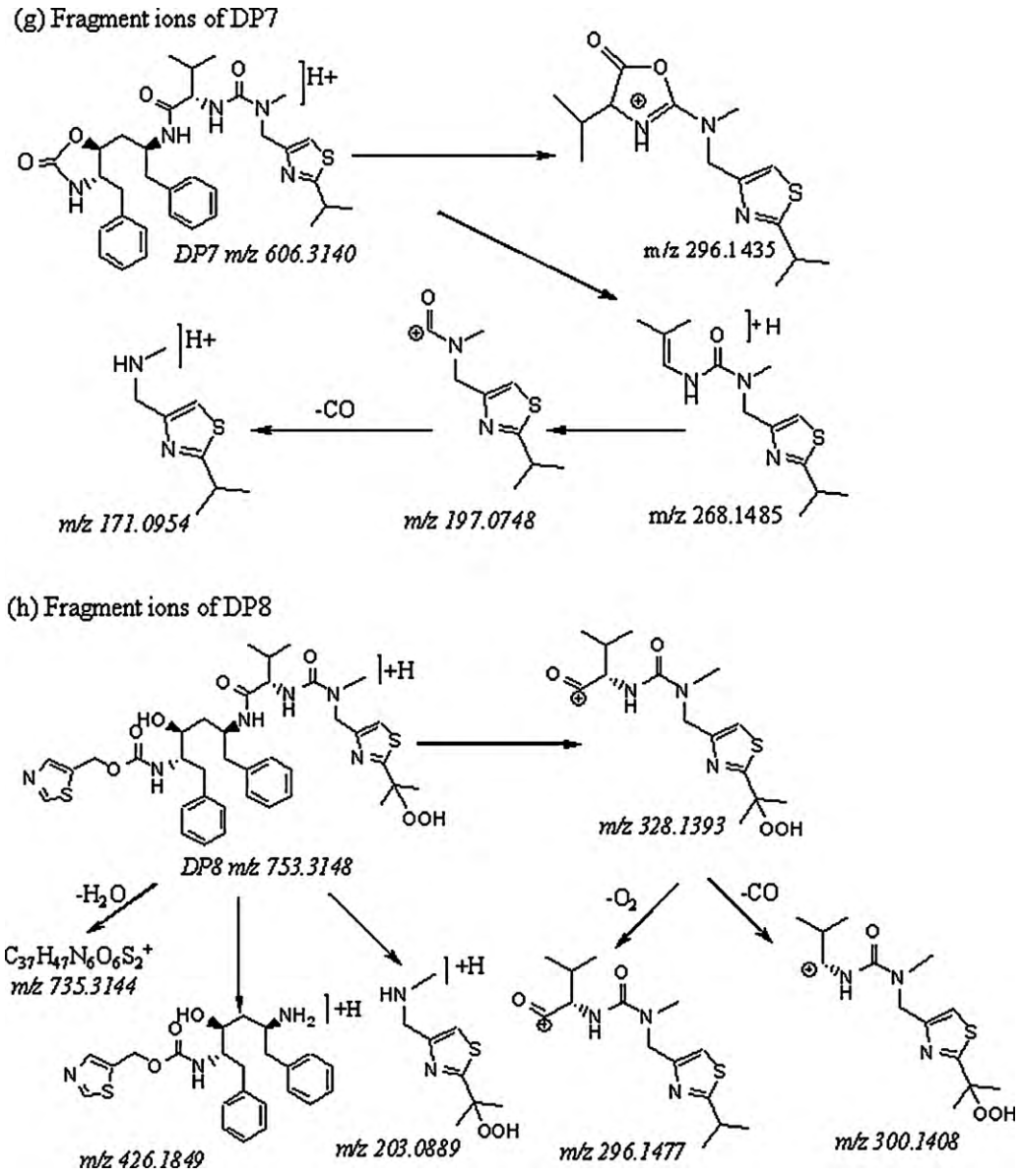

Fig. 5. (*Continued*).

The degradant DP5 (*m*/*z* 436.2278 Da) was generated from DP6 by cyclization at urea site. The fragment ion *m*/*z* 143 supported presence of cyclic ring at urea site. Also, the mass difference 44 amu between precursor ion and fragment ion 392 revealed, the loss of CO_2 molecule, which intern supported presence of cyclic ring at carbamate site (Figs. 5 and 6). Another degradant DP6 (171.0954 Da) was identified as (2-isopropylthiazol-4-yl)-N-methylmethanamine and it was formed during conversion of DP7 to DP5. It has only one fragment ion at *m*/*z* 140 (Fig. 5). The neutral loss of (thiazole-5-yl)methanol followed by dehydration from precursor ion gave DP7 (*m*/*z* 606.3140 Da). Its suggested chemical formula was $C_{33}H_{44}N_5O_4S^+$. The fragment ions at *m*/*z* 296, 268 and 171 were similar to that of RTV (Figs. 5 and 6). The accurate mass *m*/*z* 753.3148 Da of DP8 was obtained by HRMS. The extra mass of 32 amu from precursor ion indicated that there might be an addition of peroxide ion, methanol or sulphur. If drug undergoes esterification with cosolvent methanol, the degradant mass should be less than precursor ion, but the higher mass 32 amu indicated that esterification not happened. The only option was addition of a peroxide ion. The fragment ion at *m*/*z* 203 revealed the attack of peroxide ion at isopropyl moiety. The fragment at *m*/*z* 296 was formed from *m*/*z* 328 fragment by the loss of 32 amu supported the addition of peroxide ion to drug molecule (Fig. 5). Taking into consideration of these facts, the structure of DP8 was confirmed. Further, the structure of DP8 was matched with impurity G in British and European Pharmacopoeia [15–17].

4.7. Degradation path way and mechanism

The structural elucidation of degradation products revealed two susceptible sites, viz., carbamate and urea moiety. The carbamate bond and amino group in the urea moiety were susceptible to hydrolytic cleavage. The formation of protonated amine in acidic and basic conditions was explained based on the previous reports in the literature [13,14]. A similar mechanism explains the formation of DP1, DP2 from the drug. During hydrolysis, the nucleophilic attack of water on the α-carbon of amine takes place, resulting in the cleavage of C–N bond to produce protonated amine.

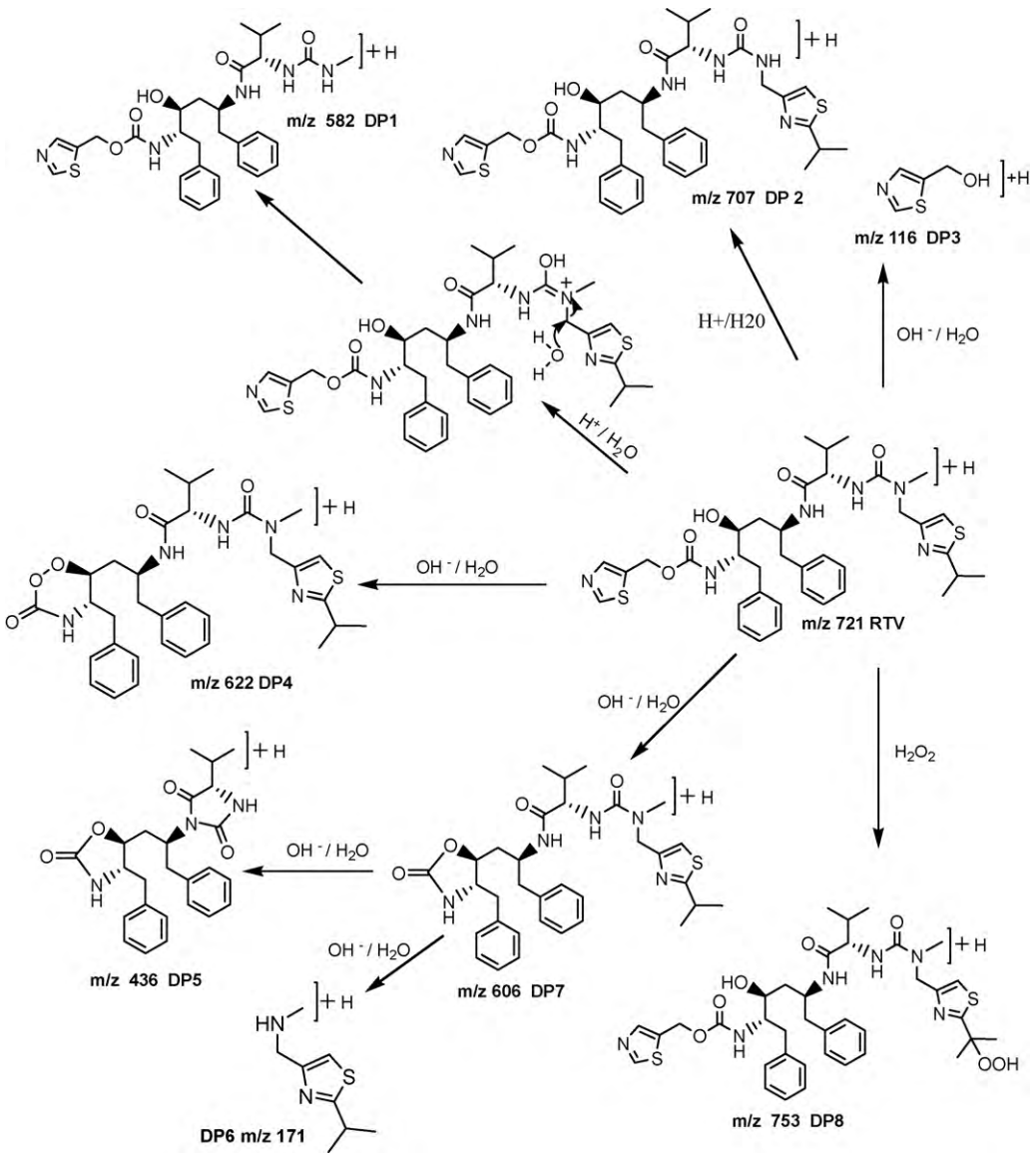

Fig. 6. Degradation pathway of ritonavir (RTV) in acidic, basic and neutral hydrolysis, oxidation, thermal and photolysis.

The hydrolysis of carbamate bond from drug molecule resulted to alcoholic moiety (DP3), and remaining carboxylic part underwent dehydration resulted to cyclic product DP7. The degradant DP5 was generated from DP7 due to again cyclization at urea derivative site. The oxidative degradation product DP8 was formed by the attack of hydrogen peroxide at isopropyl group in thiazole moiety.

5. Conclusions

A validated stability indicating assay LC-PDA method was developed to study the degradation behavior of ritonavir under hydrolysis (acid, base and neutral), oxidation, thermal and photolysis conditions. LC–MS/MS characterization of degradation products was carried out and pathways of decomposition were proposed. The drug was found to be degraded extensively in all conditions except oxidation and photolysis due to the presence of carbamate and urea linkages, which were susceptible to hydrolysis.

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