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Separation and characterization of forced degradation products of abacavir sulphate by LC–MS/MS

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ABSTRACT

Abacavir sulphate was subjected to forced degradation under the conditions of hydrolysis (acid, alkali and neutral), oxidation, photolysis and thermal stress as prescribed by ICH. Eight degradation products were formed and their separation was accomplished on Waters XTerra C_{18} (250 mm × 4.6 mm, 5 μm) column using 20 mM ammonium acetate:acetonitrile as a mobile phase in gradient elution mode by LC. The degradation products were characterized by LC–MS/MS and its fragmentation pathways were proposed. No previous reports were found in the literature regarding the degradation behavior of abacavir sulphate.

1. Introduction

Abacavir sulphate (ABC) (1S, cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9yl]-2-cyclopentene-1-methanol sulphate (Fig. 1) is one of the nucleoside reverse transcriptase inhibitors (NRTIs) used to treat acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) [1], the activity of the drug is caused by the intracellularly formed carbovir triphosphate. Formulations of abacavir sulphate are sold under the trade name of Ziagen for oral administration. Their stability is greatly influenced by varied environmental conditions such as temperature, light and humidity. A very few reports are available in the literature on degradation of abacavir sulphate. A few HPLC and LC–MS methods for the determination of abacavir sulphate in plasma, human serum and other biological matrices were reported [2–4]. Simultaneous determination of abacavir sulphate and other antiretrovirals in pharmaceutical dosages was also carried out [5–11]. Seshachalam et al. have studied its forced degradation by HPLC [12]. However, neither the extent of degradation nor the characterization of degradation products was reported. The monograph published by World Health Organization (WHO) reported six impurities, but their classification into process related and degradation products was not described [13]. The complete degradation profile of ABC and the mechanism of its degradation products are not yet reported in the literature. Tandem mass spectrometry (MS^n) and LC coupled with mass spectrometry (LC–MS, LC–MS/MS) are becoming the most versatile techniques for characterization of pharmaceutical degradation products and impurity profiling [14]. The present manuscript describes the (i) degradation behavior of abacavir sulphate under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions, (ii) optimization of LC conditions to separate the drug and its degradation products on a reversed-phase C_{18} column, (iii) method validation, (iv) characterization of degradation products and (v) fragmentation pathways of degradants using LC–MS/MS.

2. Experimental

2.1. Chemicals and reagents

Abacavir sulphate (99% purity) was a gift sample from a local manufacturing unit in Hyderabad, India. Glass-distilled and de-ionized water (Nanopure, Bransted, USA) was used. HPLC grade acetonitrile was purchased from Rankem (Mumbai, India). Analytical reagent grade sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from S.D. Fine Chemicals (Mumbai, India). 2,2′-azobisisobutyronitrile (AIBN) was also purchased from S.D. Fine Chemicals (Mumbai, India).

2.2. Instrumentation

The 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,

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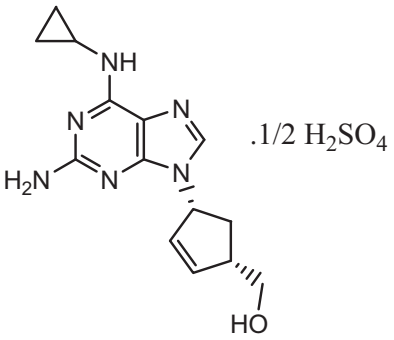

Fig. 1. Chemical structure of abacavir sulphate.

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 LCsolutions data acquiring software (Shimadzu, Kyoto, Japan). LC–MS/MS was performed by Agilent1100 series online ion trap MSD mass spectrometer with APCI source in positive mode equipped with an autosampler (G1329A), and diode array detector (G1315B) (all from Agilent technologies, Waldbronn, Germany). The data 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

Forced degradation of ABC was carried out according to ICH guidelines Q1A (R_2) [15]. About 10.0 mg of ABC was subjected to forced degradation under acidic, basic and neutral conditions by refluxing with 10.0 mL of 1N HCl, 1N NaOH and water at 80 °C for 24, 48 and 72 h respectively. Oxidation of ABC (1.0 mg/mL) was carried out using 3%, 6% H_2O_2 and 1.5 mM AIBN (2,2′-azobisisobutyronitrile) for 7 days. The ABC substance was spread to about 1.0 mm thickness in a Petri dish and kept at 100 °C for 10 days for thermal and exposed to UV light at 320 nm for 60 h for photolytic stress. After completion of stress, all the collected samples were kept in refrigerator at 5 °C.

2.4. Sample preparation

The degradation products of acid and base hydrolysis were neutralized with sodium hydroxide and hydrochloric acid respectively. The samples were further diluted to 10 times with de-ionized water. Other products of degradation viz., thermal, photolysis, oxidation and neutral hydrolysis were diluted to 10 times with water. All the samples were filtered through 0.22 μm membrane filter before HPLC analysis.

2.5. Chromatographic conditions

All the samples were analyzed by HPLC on a Waters XTerra, C_{18} column using 20 mM aqueous ammonium acetate:acetonitrile as a mobile phase in gradient elution at a flow rate of 1.0 mL/min at ambient temperature. The detection was carried out by photodiode array detector (PDA) at 220 nm.

2.6. Mass spectrometric conditions

The mass spectra were recorded in atmospheric pressure chemical ionization (APCI) in positive mode of detection. Nitrogen was the nebulizer and curtain gas. Collision-induced dissociation was achieved by helium as a collision gas. The ion source conditions were set as follows: dry temperature, 325 °C; nebulizer gas, 60 psi; dry gas, 5.0 L/min; capillary exit, 113.5 V; capillary current, 81.787 nA; corona current, 4000 nA; electro multiplier voltage, 2100 V; vaporizer temperature, 400 °C and dwell time, 200 ms. The HRMS data 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.

3. Results and discussion

3.1. Optimization of chromatographic conditions

During the optimization process, preliminary experiments were carried out on Waters XTerra, C_{18} column (250 mm × 4.6 mm, 5 μm) using water:acetonitrile (90:10, v/v) as a mobile phase. The peaks corresponding to degradation products did not resolve completely and tailing was noticed. To get acceptable separation between the drug and its degradation products, ammonium acetate buffer was used instead of water. Aqueous ammonium acetate buffer (20 mM):acetonitrile in gradient elution mode was successful for separation of the ABC and its degradation products. The optimized gradient elution programme is shown in Table 1. The flow rate was 1.0 mL/min and detection wavelength was 220 nm. The runtime was 25.0 min. The optimized chromatographic conditions were used for separation of ABC and its degradation products. The method was validated with respect to the parameters outlined in ICH guidelines Q1A (R_2) and extended to LC–MS/MS.

3.2. Validation

3.2.1. Specificity

Specificity is the ability of the analytical method to measure the analyte concentration accurately in presence of all the potential impurities. The specificity was determined by subjecting API to stress under various conditions. All the degradation products were well separated from the API and the method was found to be specific.

3.2.2. Linearity

Good linearity was observed in the concentration range 0.5–10.0 μg/mL of API. The data was subjected to statistical

Table 1
Gradient elution programme.

Time (min)	Mobile phase	
	A (%)	B (%)
0.01	95.0	5.0
10.00	85.0	15.0
15.00	40.0	60.0
20.00	10.0	90.0
20.01	95.0	5.0
25.00	95.0	5.0

A, 20 mM aqueous ammonium acetate; B, acetonitrile.

Table 2
Intra- and inter-day precision data.

Conc. (μg/mL)	Measured conc. (μg/mL) ± RSD (%)	
	Intra-day	Inter-day
2.0	2.3203 ± 1.3187	2.3004 ± 1.3302
5.0	5.2237 ± 0.6377	5.2217 ± 0.6377
10.0	10.0725 ± 0.3437	10.0749 ± 0.3431

$n = 5$.

analysis using a linear regression model; the linear regression equation and correlation coefficient (r^2) were $Y = 55{,}868X + 15{,}285$, >0.9960 respectively. The results have indicated a good linearity. The limits of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield a signal-to-noise ratio of 3 for LOD and 10 for LOQ respectively. The LOD and LOQ values were found to be 20 and 60 ng/mL respectively.

3.2.3. Accuracy and precision

Intra- and inter-day precision and accuracy was assessed using three quality control samples. Five replicates were analyzed everyday to determine the intra-day accuracy and precision. The procedure was repeated three times over 3 days in order to determine the inter-day accuracy and precision. Good precision and accuracy was observed. The intra- and inter-day precision data is given in Table 2.

3.3. Degradation behavior

Fig. 2 shows the typical chromatograms of the degradation products formed under a variety of stress conditions. The chromatographic parameters i.e., retention times, resolution, tailing factor and peak purity were determined and given in Table 3.

3.3.1. Hydrolysis

Under acid hydrolysis, the drug degraded completely resulting in five degradation products (A_1–A_5). However, no degradation was observed under base and neutral hydrolysis. The degradation products of acid hydrolysis were analyzed by LC–MS and the chromatographic data is shown in Table 3.

3.3.2. Oxidation

The drug was oxidized using 3% H_2O_2, 6% H_2O_2 and 1.5 mM AIBN for 7 days. It was found that, 3% H_2O_2 was ineffective in oxidizing the drug even after 7 days, whereas 6% H_2O_2 could degrade it after 7 days at ambient temperature. Under these conditions, two degradation products (O_2 and O_3) were formed. One more oxidative degradent O_1 was formed along with O_2 in the presence of 1.5 mM AIBN [16] at 45 °C after 7 days.

3.3.3. Photolytic and thermal degradation

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

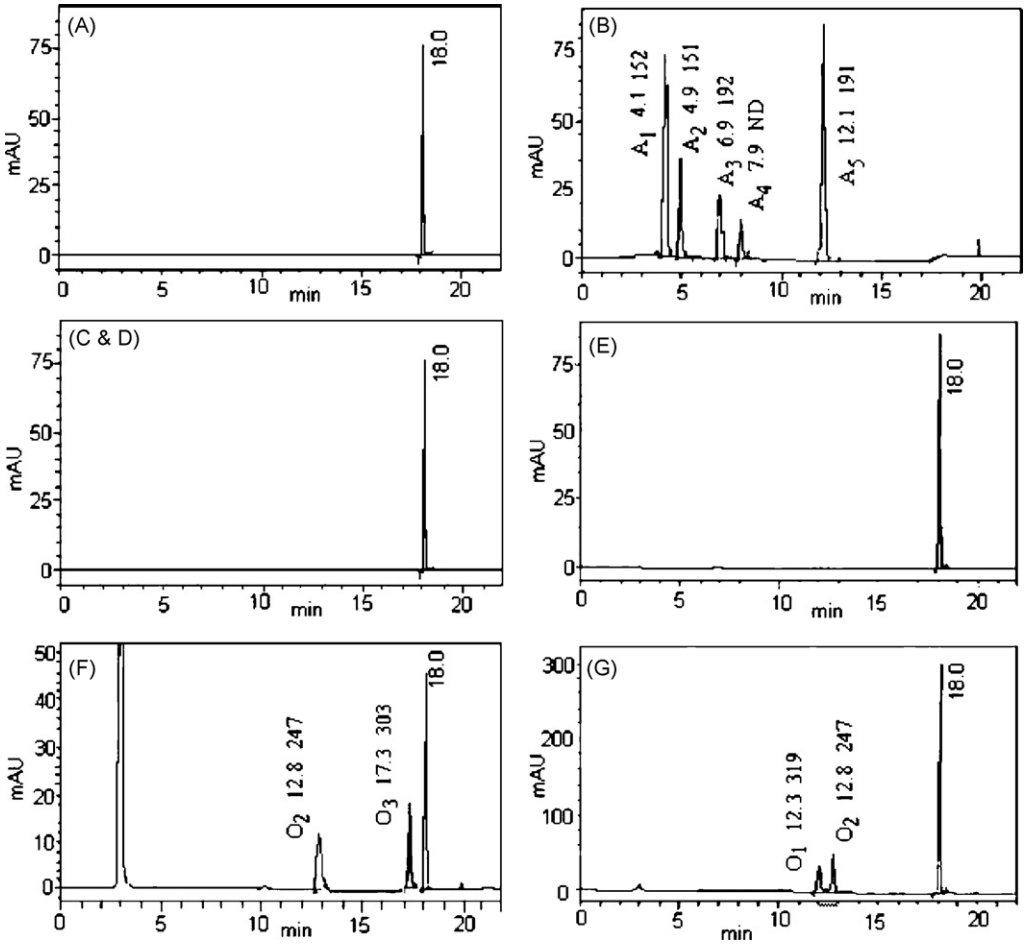

Fig. 2. Typical HPLC chromatograms of: (A) abacavir sulphate and its degradation products under (B) acid hydrolysis, (C) base hydrolysis, (D) neutral hydrolysis, (E) photolysis, (F) 6% hydrogen peroxide, and (G) AIBN.

Table 3
Peak purity and chromatographic data of degradation products.

Degradation product	Retention time (R_t) (min)	P.P.I	S.P.T	Tailing factor (T_f)	Resolution (R_S)
A1	4.1	0.9993	0.9965	1.22	2.3
A2	4.9	0.9999	0.9967	1.19	3.5
A3	6.9	0.9938	0.9338	1.11	6.5
A4	7.9	0.9999	0.9983	1.20	2.7
A5	12.1	0.9999	0.9993	1.17	12.7
O1	12.4	0.9995	0.8401	1.19	6.2
O2	12.8	0.9999	0.9671	1.13	8.8
O3	17.3	0.9995	0.9048	1.22	17.8

P.P.I, peak purity index; S.P.T, single point threshold.

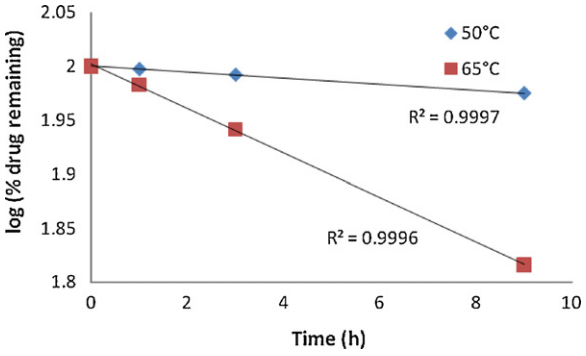

Fig. 3. First-order plots for the degradation of abacavir sulphate in acid hydrolysis at (◆) 50 °C and (■) 65 °C.

3.4. Kinetics of degradation

The samples collected at different intervals of time under various stress conditions were analyzed by HPLC. Stress conditions such as oxidation, photolysis, base and neutral hydrolysis were found to be not effective even after 24.0 h. However, on acid hydrolysis ABC was degraded rapidly and completely within 3.0 h. Hence the kinetics of degradation of the ABC under acid hydrolysis was studied.

To perform kinetic studies the temperature of acid hydrolysis was altered to 65 and 50 °C. A series of stressed samples were collected at 0.5, 1.0, 2.0, 3.0, 6.0, 9.0, and 12.0 h of acid hydrolysis and analyzed by HPLC. Then the chromatographic data revealed that only two degradation products (A_2 and A_5) were formed, where A_5 as major product. The plots of log (% of drug remaining) versus time in the course of the kinetic studies are shown in Fig. 3.

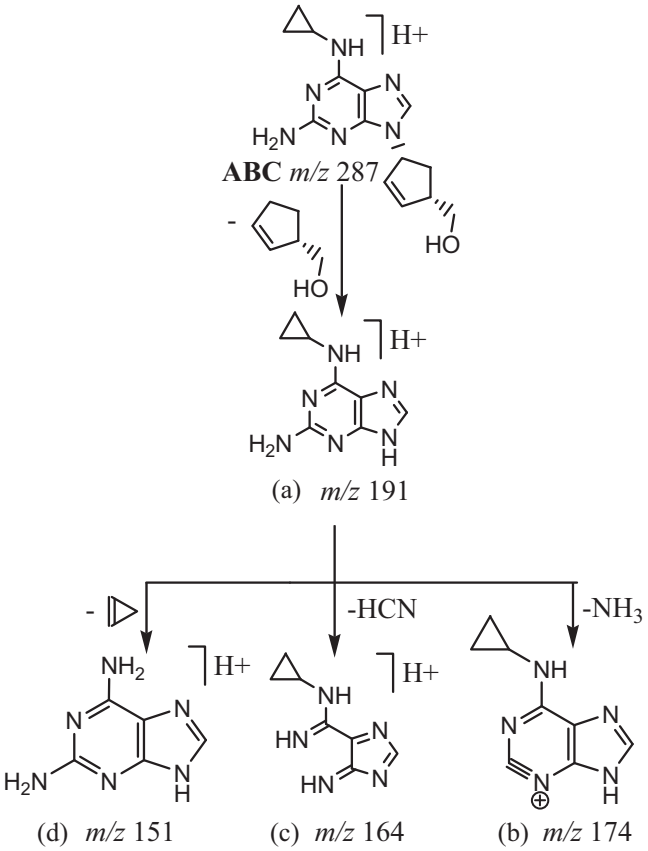

Fig. 5. MS^n fragmentation pathway of abacavir sulphate.

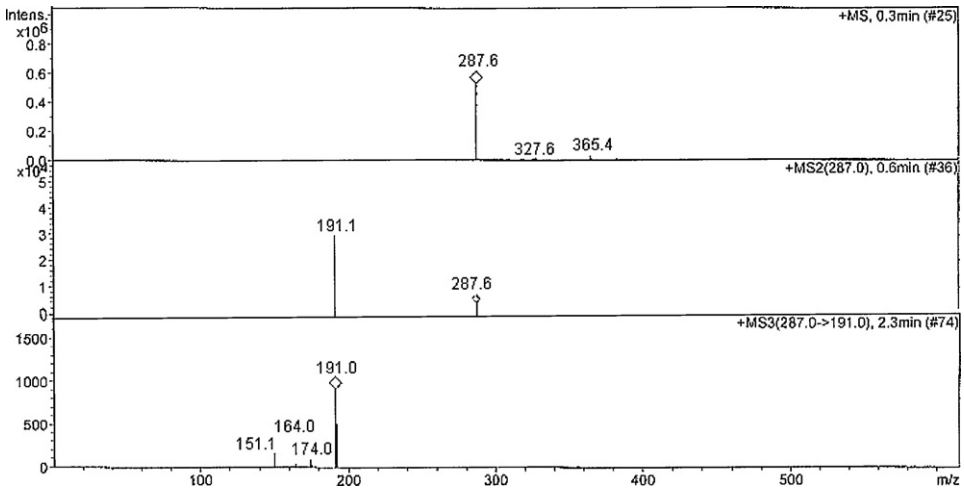

Fig. 4. MS^n fragmentation mass spectra of abacavir sulphate ($n = 3$).

Table 4
Degradation rate constant (k), half-life ($t_{1/2}$) and t_{90} for ABC under acid hydrolysis at 50 and 65 °C.

Temperature (°C)	k_{obs} (h^{-10})	$t_{1/2}$ (h)	t_{90} (h)
50.0	0.0064	108.28	359.84
65.0	0.0474	14.60	48.58

At the selected temperature the degradation process of ABC could be described by apparent first-order kinetics. At temperatures 50 and 65 °C, strict straight line behavior (correlation coefficient, $r^2 > 0.999$) was observed. It confirms the degradation process was first-order kinetic model. From the slopes of the straight lines it was possible to calculate the apparent first-order degradation rate constant k, half-life ($t_{1/2}$), and time required for 90% degradation (t_{90}) at each temperature (Table 4). The rate constant was increased from 0.0064 h^{-1} at 50 °C to 0.0474 h^{-1} at 65 °C.

3.5. MS^n study of abacavir sulphate

The MS^n spectra of ABC are shown in Fig. 4. At lower collision energy 0.2 mA, protonation of the drug took place and the molecular ion peak at m/z 287 was observed. At high collision energy 0.45 mA, the elimination of cyclopentenyl moiety from 9-N atom of purine ring was observed by the formation of corresponding protonated adenine ion at m/z 191. Similar results could be found in the literature for nucleobases and related synthetic substrates [17,18]. The MS^3 studies at collision energy 0.55 mA, revealed that the ion at m/z 191 underwent further fragmentation gave three product ions at m/z 151, 164 and 174. The former ion was formed due to the loss of cyclopropene [19], whereas the later ions were generated by the loss of neutral molecules viz., HCN and NH_3 respectively [20]. The mass spectral fragmentation pathway of ABC is summarized in Fig. 5 The HRMS data also supported the fragmentation profile of ABC (Table 5).

3.6. Characterization of degradation products

3.6.1. Acid hydrolysis

Abacavir sulphate, on acid hydrolysis yielded five degradation products. Table 6 gives the m/z values of the degradants and its fragmentation ions. The proposed fragmentation pathways for the degradation products of ABC in acidic condition are depicted in Fig. 6. The observed and theoretical accurate mass values for degradation products, along with error in ppm and determined molecular formula of each are given in Table 7. It starts with the cleavage of bond between cyclopentenyl moiety and 9-N atom of purine ring resulted to degradant A_5 at m/z 191.1442 Da. The combination of fragment ions 134 and 58 resulted in A_5 (Fig. 6). The same

Table 5
HRMS data of ABC and its fragment ions.

Fragmention	Observed ion mass m/z (amu)	Best possible molecular formula	Theoretical mass m/z (amu)	Error (ppm)	RDB
$[M+H]^+$	287.1516	$C14H19N6O^+$	287.1563	0.16	8.0
a	191.1141	$C8H11N6^+$	191.1129	−6.27	6.0
b	174.0215	$C8H8N5^+$	174.0246	0.17	7.0
c	164.0615	$C7H10N^+$	164.0612	−1.82	5.5
d	151.0526	$C5H7N6^+$	151.0502	−0.15	5.0

RDB, rings plus double bonds.

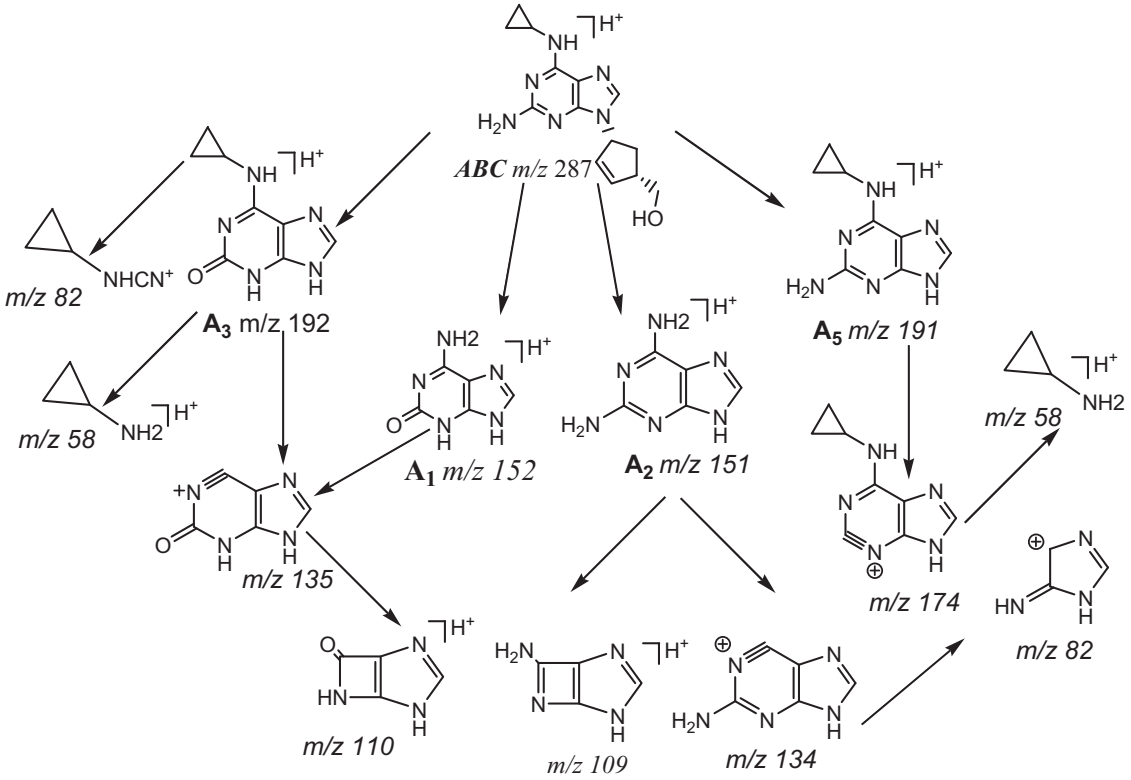

Fig. 6. Proposed fragmentation pathway of degradation products of ABC under acid hydrolysis.

Table 6
m/z values of degradants and its fragment ions of ABC.

Degradation product	Molecular ion *m/z* (amu)	Fragment ions *m/z* (amu)
A_1	152	135, 110
A_2	151	134, 109
A_3	192	134, 109, 82, 58
A_4	ND	–
A_5	191	174, 151, 134, 109, 82, 58
O_1	319	223, 205, 151, 134, 79
O_2	247	151, 134, 79
O_3	303	207, 191, 67

ND, not detected.

was supported even by their elemental composition, calculated from accurate masses, as $C_8H_{11}N_6^+$. The molecular ion peak A_2 (151.0679 Da) formed by hydrolysis at cyclopropyl ring from A_5 leads to the formation of 2-amino adenine. The fragment ion at *m/z* 134 formed from A_2 by the loss of 17 amu revealed that the precursor ion contains amine group. It followed same fragmentation pattern as the degradation product A_1. In case of A_3, the experimental *m/z* value was 192.0879 Da and its suggested elemental composition was $C_8H_{10}N_5O^+$. Its formation may be best explained by an attack of water molecule on the NH_2 group of purine moiety followed by the elimination of NH_3 [14,15]. The CID MS^2 of *m/z* 192 ion gave *m/z* 135 (loss of cyclopropyl amine), and *m/z* 58 (cyclopropyl amine cation) ions. The accurate mass measurement data and the formation of *m/z* 135 and *m/z* 58 ions may also supports the structure of A_3. Another degradation product A_1 (152.0566 Da) formed by hydrolysis at cyclopropyl ring from A_3 leads to the formation of 6-amino-3H-purin-2(9H)-one. Its suggested chemical formula was $C_5H_6N_5O^+$. The identical fragment ions at *m/z* 135 and 110 revealed that the degradant A_1 was the primary degradant pair of A_3. According to nitrogen rule the precursor ion (A_1) at *m/z* 152 should have odd number of nitrogen atoms. The peak A_4 at retention time 7.9 min was not detected by LC–MS, possibly due to poor ionizability of the molecule. All the above

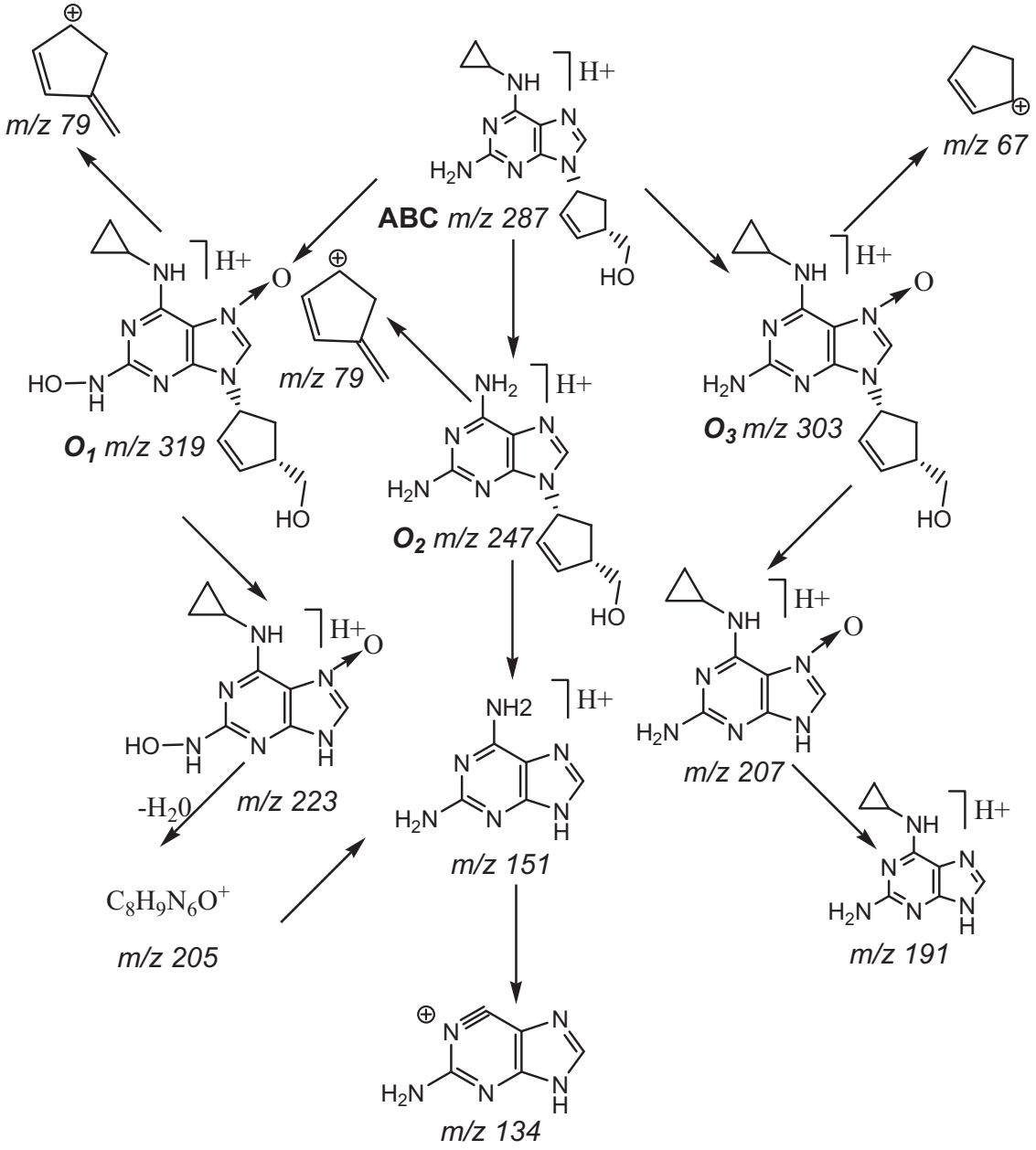

Fig. 7. Proposed fragmentation pathway of degradation products of ABC under oxidation.

Table 7
HRMS data of degradation products of abacavir sulphate.

Degradation product	Theoretical mass *m*/*z* (amu)	Most probable molecular formula	Observed mass *m*/*z* (amu)	Error (ppm)	RDB
A_1	152.0566	C5H6N5O+	152.0534	−2.10	5.0
A_2	151.0679	C5H7N6+	151.0674	−0.30	5.0
A_3	192.0879	C8H10N5O+	192.0846	−1.71	6.0
A_4	ND	–	–	–	–
A_5	191.1442	C8H11N6+	191.1425	−0.88	6.0
O_1	319.1513	C14H19N6O3+	319.1515	0.62	9.0
O_2	247.1301	C11H15N6O+	247.1307	2.42	7.0
O_3	303.1564	C14H19N6O2+	303.1567	0.98	9.0

RDB, rings plus double bonds.

degradation products were supported by the HRMS results as shown in Table 7.

3.6.2. Oxidation

The oxidation of abacavir sulphate yielded three degradation products. The degradants O_1 and O_2 were formed in the presence of AIBN at 45 °C for 7 days, while O_2 and O_3 were formed with 6% H_2O_2 at room temperature for 7 days. The oxidation product O_1 has a molecular ion at *m*/*z* 319.1513 Da. Its mass could be attributed due to the formation of N-oxide and hydroxyl amine at 7th and 2nd positions of purine moiety. The formation of N-oxide was strongly supported by previous literature [21]. The MS^2 fragment ions of O_1 were at *m*/*z* 223, 205, 151, 79 and 67. The elimination of a water molecule from *m*/*z* 223 yielded *m*/*z* 205, confirming the presence of hydroxylamine group (Fig. 7). The probable chemical formula given by HRMS is $C_{14}H_{19}N_6O_3^+$. Similarly, the degradation product O_3 also consists of N-oxide formation at 7th position of purine moiety. The fragment ion structures at *m*/*z* 207 and 191 supported the addition oxygen to purine moiety. This was confirmed by the previous reports [21,22]. The second oxidation product O_2 was observed at *m*/*z* 247. It was formed simply by the loss of a cyclopropene group. The further MS/MS fragments at *m*/*z* 207, 191, and 174 and also, the HRMS results (Table 7) supported the proposed structure. All the oxidative degradation products and their fragmentation pattern are shown in Fig. 7.

4. Conclusions

A validated LC–MS/MS method for stability indicating assay of abacavir sulphate was developed. The degradation behavior of abacavir sulphate under hydrolysis (acid, base and neutral), oxidation, photolysis and thermal stress conditions was studied. The degradation products were characterized and the fragmentation pathways were proposed based on LC–MS/MS data and HRMS results. In addition to that, the degradation behavior of ABC in acid hydrolysis was best explained by kinetic studies.

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