Detection of potential degradants in Oseltamivir by Liquid Chromatography-Tandem Mass Spectroscopy

Short title: Detection of potential degradants in Oseltamivir by LC-MS/MS

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Abstract

Objectives: A sensitive and reliable Liquid Chromatography and Mass Tandem Spectroscopy method was developed for the detection of possible degradants in Oseltamivir. The rationale behind this study was to identify the degradants present in Oseltamivir that could pose risk to patient at the far end by early detection. Degradants can appear at any stage from the starting of raw material collection through the entire process of synthesis ,manufacture ,formulation and storage. Oseltamavir is subjected to forced degradation studies like thermal and oxidative stress. **Materials and methods:** The Thermal degradation study was carried out in solid state by exposing Oseltamivir in a petri plate as a very thin layer to dry heat in the oven at 105◦C for 12 hrs. Peroxide degradation was carried out by treating the drug with 10mL of 3% Hydrogen peroxide and kept it on a bench for 2 hrs. Oseltamivir standard and test solution samples were prepared and analyzed by using LC-MS/MS method. **Results and discussion:** Liquid chromatogram showed retention time at 0.731, 0.723 and 0.714 minutes for standard and test solutions respectively. The mass spectral data are presented with a mass range of 100-600 m/z on (x-axis) and intensity on (y-axis). The obtained mass spectral data revealed that the peak intensity of API and stressed samples have no significant difference. **Conclusion:**The antiviral drug Oseltamivir remained unaffected under stress degradation conditions of both peroxide and thermal based on the observed LC-MS/MS spectral evidence, it proved that the inherent stability of the Oseltamivir under stress condition to be good.

Keywords: Oseltamavir, forced degradation,LC-MS/MS

Introduction

Oseltamivir belongs to the class of antiviral agents. It is neuraminidase inhibitor which brings about its action by inhibiting the viral neuraminidase enzyme that is present on the surface of the virus thereby preventing budding from host cell, viral replication and its ability to infect. Mainly used infections caused by influenza virus A & B.[1] The best results with oseltamivir in the treatment of influenza was clinically observed when it is administered within 48 hours of the onset of symptoms associated with influenza.[2] Oseltamivir is said to beneficial in the treatment of acute, uncomplicated illness due to influenza A and B infection in neonates and older if they have symptoms of influenza for not more than 48 hours according to FDA prescribing information and also indicated as a prophylactic against influenza virus in patients one year and older. During the influenza pandemic outbreak Oseltamivir was indicated for post-exposure prevention of influenza in infants less than 1 year of age.[3] Oseltamivir, a synthetic derivative prodrug of ethyl ester having an acetamido cyclohexene which is a structural homolog of sialic acid. Oseltamivir sold under the brand name Tamiflu.[4] From review of literature few stability indicating methods by HPLC are reported for Oseltamivir but no LC-MS/MS have been reported for forced degradation studies under thermal & oxidative stress conditions.[5-13]

The main aim was to identify the unknown degradants present in Oseltamivir drug was subjected to different stress degradations such as peroxide, and thermal conditions as per ICH Q1A (R2) recommendations and the analysis was carried out by LC-MS /MS system.[14,15]

LC-MS/MS is a well established analytical tool combining the separating power of liquid chromatography with highly sensitive and selective mass analysis capability of triple quadrupole mass spectrometry, with rapid identification and characterization of component in sample mixtures. It can also provide the structure through the fragmentation pattern of the analytes. MS analyze the precursor ion, in an ion trap, a quadrupole or time-of-flight Mass spectrometer. MS/MS is the combination of two mass analyzers in one mass spectra instrument. The first MS filters for the precursor ion followed by a fragmentation of the precursor ion with high energy and nitrogen gas. A second mass analyzer is then filtering for the product ions, generated by the fragmentation. This is usually done in a triple quadrupole MS (QQQ) or a QTOF. The

advantages of MS/MS were the increased sensitivity due to reduction of noise and can gain more structural information on analyte (QTOF) based on the fragmentation pattern, and MS measures the m/z of a compound whereas, MS/MS measures the m/z of the compound as well as its intermediates.

Materials and Methods

All chemicals and solvents used in the present study was of analytical and HPLC grade. Water of Millipore, Acetonitrile, Methanol and Hydrogen peroxide from Merck. Instruments used were LC-MS/MS (AB SCIEX 6500), pH /Ion analyzer (Metrohm, M780),Precision water bath(Julabo, Seelbach)and dry-bath (Thermolyne,IA)

Sample preparation for forced degradation study

The Thermal degradation study was carried out in solid state by exposing Oseltamivir in a petri plate as a very thin layer to dry heat in the oven at 105◦C for 12 hrs. Peroxide degradation was carried out by treating the drug with 10mL of 3% Hydrogen peroxide and kept it on a bench for 2 hrs.

Procedure for Thermal degradation:

Thermal degradation study was carried out in solid state by exposing Oseltamivir in a petri plate with a very thin layer to dry heat in the oven at 105oC for 12 hrs. Thermally stressed 10 mg sample was dissolved in 1ml of DMSO in a 10 ml volumetric flask and the solution was made upto 10 ml with methanol (1mg/ml). From the above solution, 1 ml of solution was pipette out and volume was made up to mark with diluent methanol to give a concentration of 1µg/ml and injected into the LC-MS/MS system.

Procedure for Peroxide degradation:

Accurately weighed 100 mg Oseltamivir was taken in a 100 ml of volumetric flask, to which about 5 ml of 3% hydrogen peroxide was added and kept it on a bench for 1 hr. Followed by solution was diluted to 100 ml with water and mixed well. From the above solution, 1 ml of solution was pipette out, added two drops of DiMethylSO in a 10 ml volumetric flask and the volume was made up to mark with diluent methanol to give a concentration of 1µg/ml and injected into the LC-MS/MS system.

Preparation of standard solution (API)

Accurately weighed 10 mg of Oseltamivir was dissolved in DMSO in a 10 mL volumetric flask and the solution was made upto 10 mL with methanol (1mg/mL) . From

the above solution, 1 mL of solution was pipette out and volume was made up to mark with diluent to give a solution containing 1µg/mL concentration of the solution and injected into the LC-MS/MS system.

LC- MS/MS Chromatographic conditions

Mobile phase A	0.1% formic acid
Mobile phase B	Acetonitrile
Method composition	Isocratic (25: 75)
Column	Eclipse Plus C18 4.6×150mm, 3.5µm
Temperature	30o C
ƛmax	240 nm
Flow	0.5ml/min
Injection volume	20µl

Model	QT of LC-MS
Probe	ESI +ve
Scan Range	700-2000 m/Z
Capillary (V)	3500
Collision energy	40
Nebulizer gas (psig)	40
Drying gas temp	300o C
Dry gas flow rate	10 L/min

Results of degradation studies

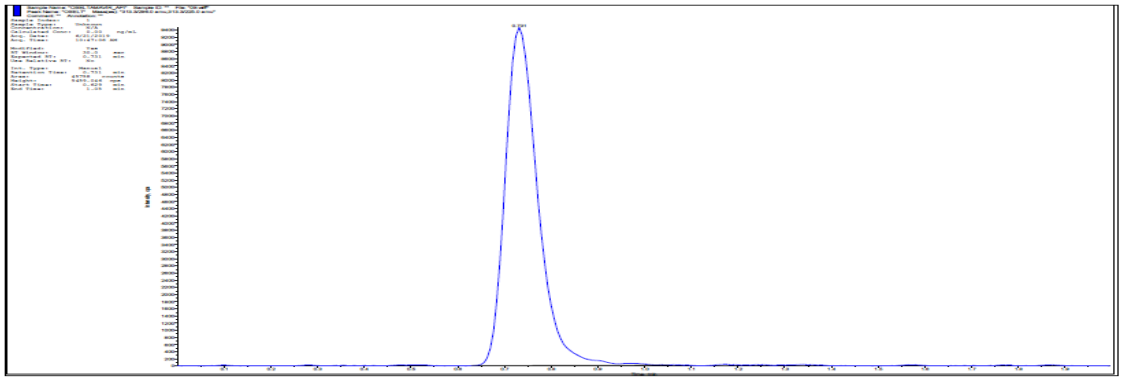


Fig 1: Oseltamivir API chromatogram

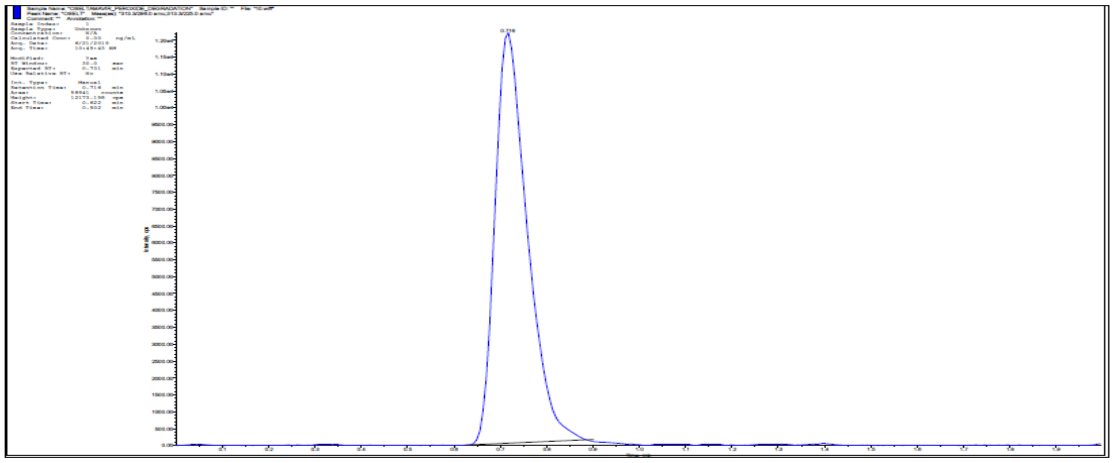


Fig 2: Oseltamivir Thermal stressed chromatogram

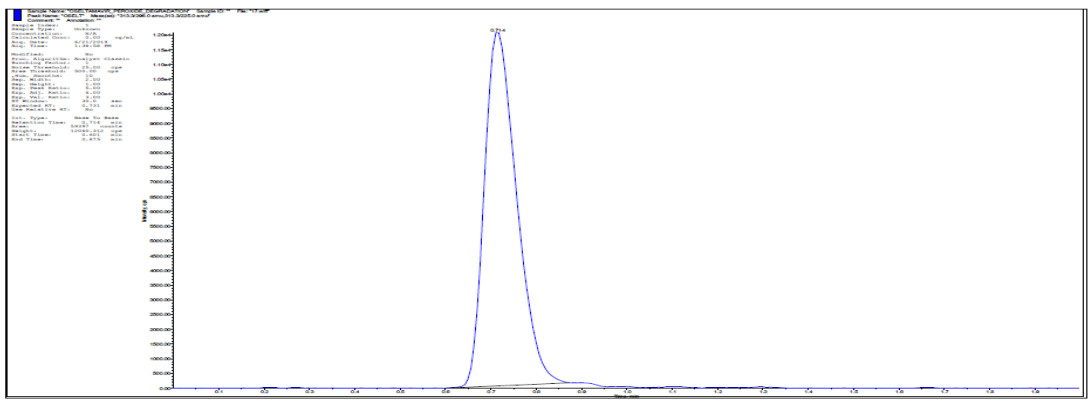


Fig 3:Oseltamivir Peroxide stressed chromatogram

Table 1: The Retention time and Analyte Peak Area (counts) of the API and stressed sample solution are given in the table below.

API	Retention time (min)	Analyte Peak Area (counts)
Oseltamivir	0.731	51307
Stress condition of Oseltamivir		
Thermal stress	0.723	58941
Peroxide stress	0.714	59397

The mass spectral data are presented with mass range 100-600 m/z on (x axis) and intensity on (y axis). The obtained mass spectral data revealed that the peak intensity of API and stressed samples have no significant difference as shown in fig 4-7 followed by the probable fragmentation pattern based on the molecular weight.

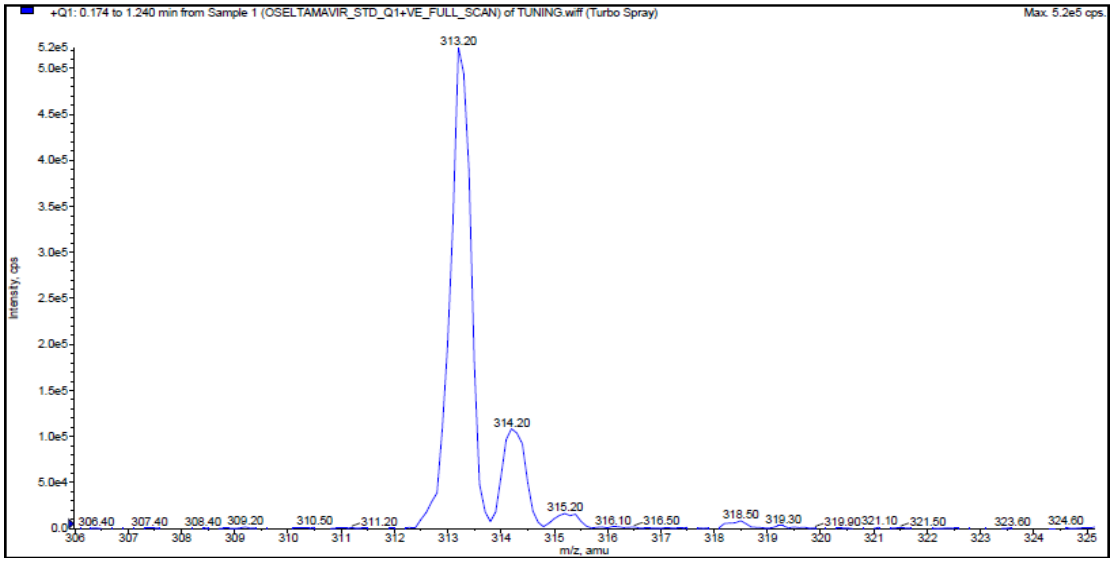


Fig 4. Mass spectra of Oseltamivir API

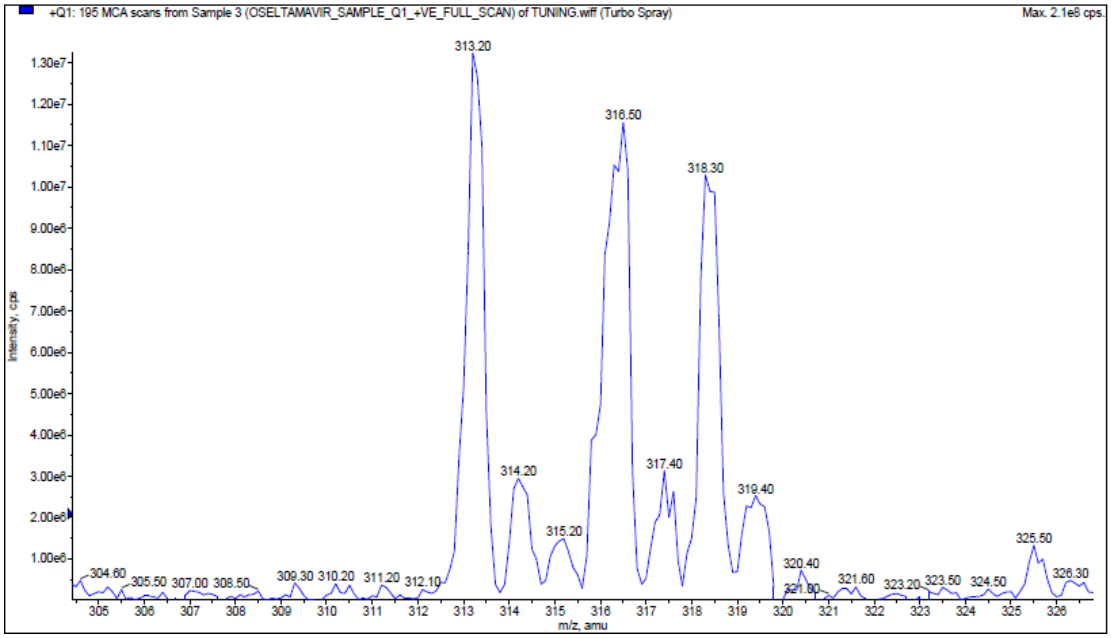


Fig 5. Mass spectra of stressed sample

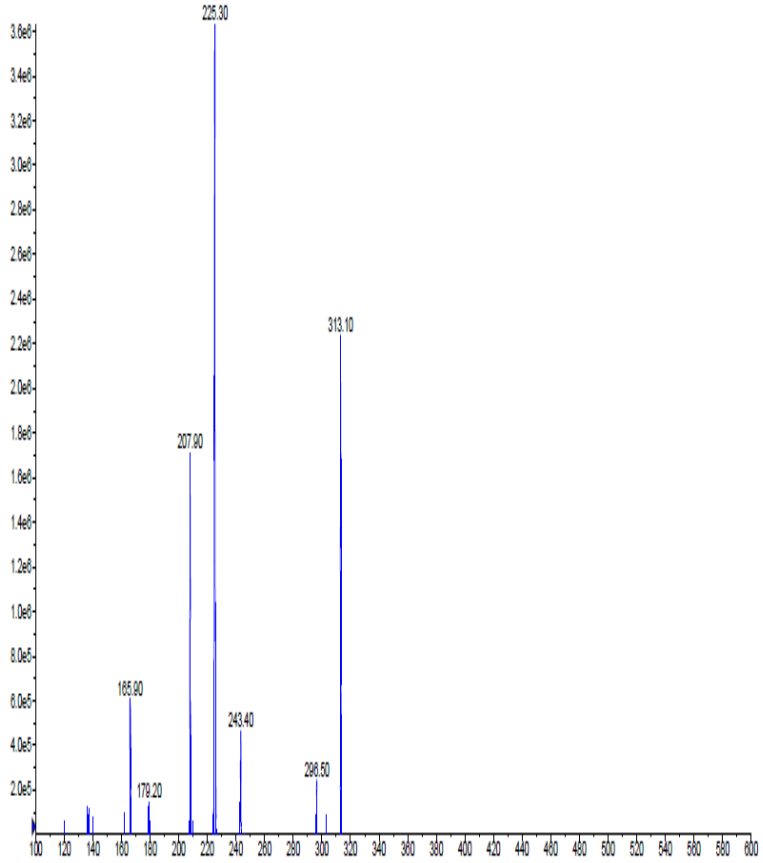


Fig 6. Fragmentation pattern of Oseltamivir API

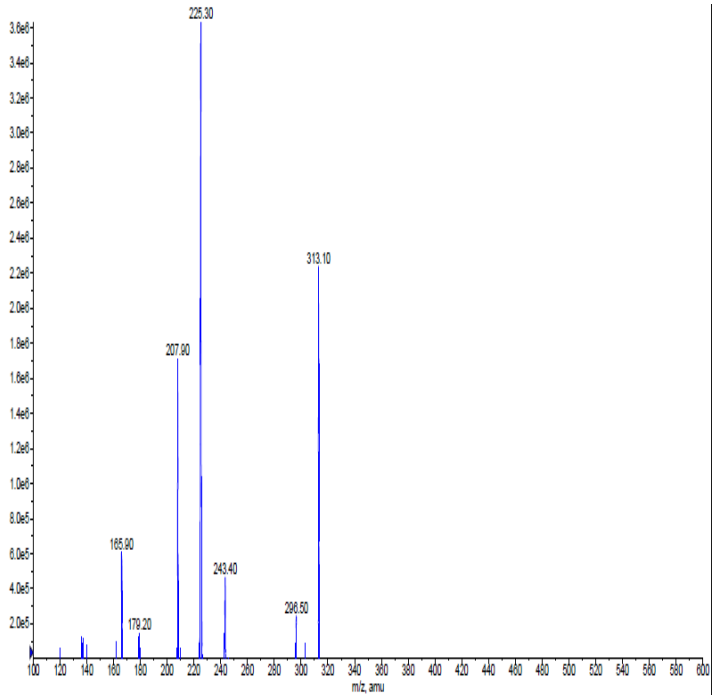
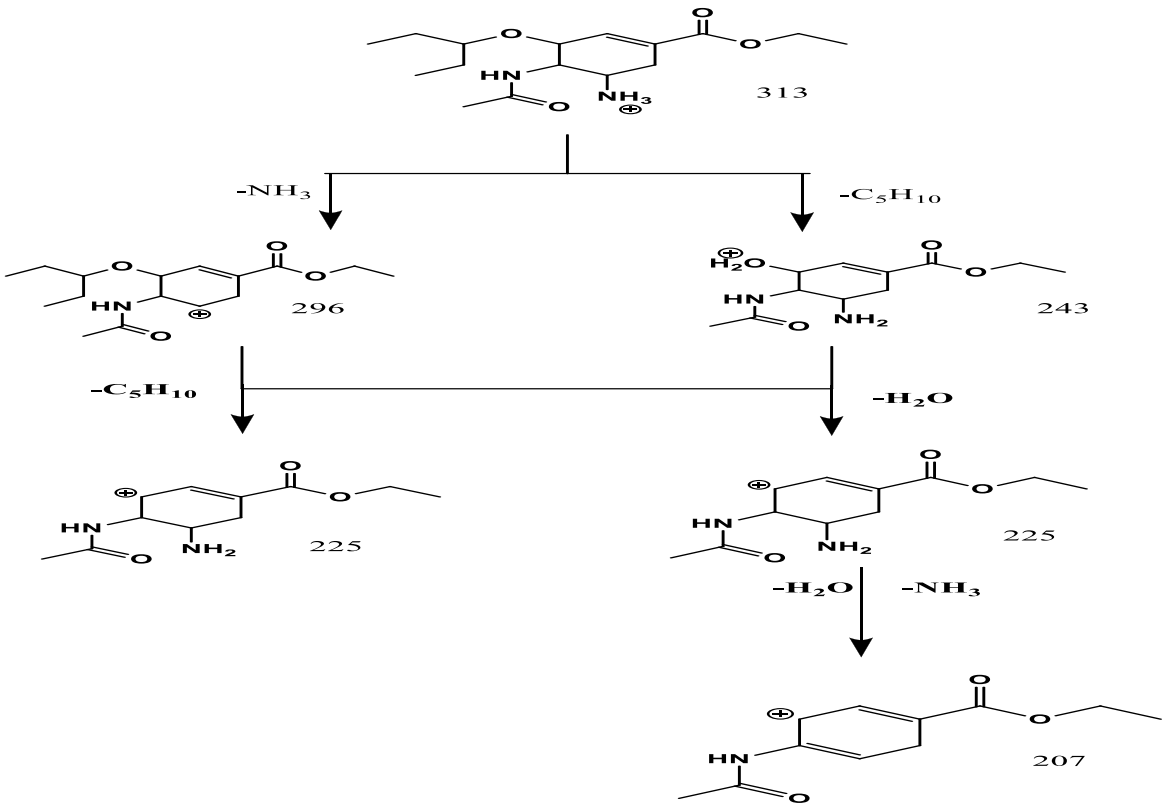
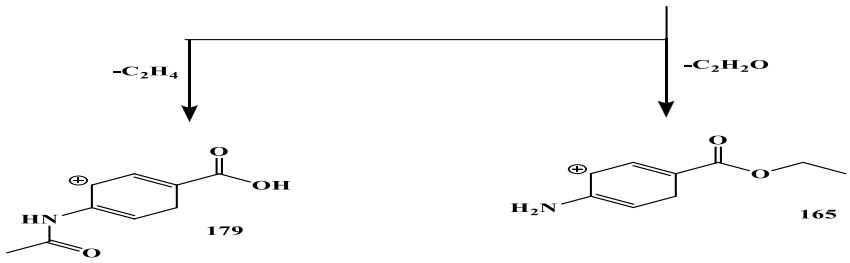


Fig 7. Probable Fragmentation pattern of stressed sample

Fig 8: Probable Fragmentation pathway of the stressed sample along with the exact masses of the fragments





Discussion

Oseltamivir standard and test solution samples were prepared and analyzed by using LC-MS/MS method. Liquid chromatogram showed retention time at 0.731, 0.723 and 0.714 minutes for standard and test solutions respectively. The mass spectral data are presented with a mass range of 100-600 m/z on (x-axis) and intensity on (y-axis). The obtained mass spectral data revealed that the peak intensity of API and stressed samples have no significant difference. The molecular ion peak of Oseltamivir (m/z 313) fragmented into product ions of m/z 296 and m/z 243, upon neutral loss of NH_3 and C_5H_{10}, respectively. While the fragment of m/z 296 further ionized to product ion of m/z 225 on the loss of C_5H_{10}, the ion of m/z 243 also fragmented further into product ions of m/z 225 by the elimination of H_2O. The fragment ion m/z 207 may be formed by the fragmentation of m/z 225 by the loss of NH_3 or H_2O.The fragment ion m/z 207 further fragmented into m/z 179 and m/z 165 may be due to the elimination of C_2H_4, and C_2H_2O.

Conclusion

Oseltamivir drug was subjected to thermal and peroxide degradation conditions, according to international conference on Harmonization guideline (ICH) Q1 A(R2) by LC-MS/MS method. The obtained LC data revealed that the retention time of Oseltamivir and stressed samples have no significant difference in API and stressed samples. The mass spectra and probable fragmentation pattern of Oseltamivir also revealed the stability of API and stressed samples. The antiviral drug Oseltamivir remained unaffected under stress degradation conditions of both peroxide and thermal based on the observed LC-MS/MS spectral evidence, it proved that the inherent stability of the Oseltamivir under stress condition to be good.

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