Intrinsic Stability Study and Forced Degradation Profiling of Olopatadine hydrochloride by RP-HPLC-DAD-HRMS Method

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ABSTRACT

Introduction: The forced degradation determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products. The objective of present research work was to establish intrinsic stability and forced degradation profiling of olopatadine hydrochloride.

Methods: The intrinsic stability of olopatadine hydrochloride has been evaluated by RP-HPLC method, where the mixture of 0.1% formic acid and organic phase (methanol : acetonitrile; 50:50 % v/v) was used as mobile phase at 1.0 mL/min in gradient mode. Different stress conditions have been employed to explore the intrinsic stability of olopatadine hydrochloride.

Results: In acidic condition, five degradation products (DPs) viz. OLO1, OLO2, OLO3, OLO4 and OLO5 were observed. OLO5 was major DP which was increased with time and peak area of OLO was decreased. In addition to OLO3 and OLO5; two more DPs were observed in alkaline condition viz. OLO6, and OLO7 in alkaline condition. OLO5 and OLO6 were two major DPs; OLO5 was increased with time while OLO6 has zig-zag pattern of peak area with time. All DPs of neutral condition were also found in acidic condition while OLO3 and OLO5 were common in all three type of hydrolytic degradation.

Discussion and Conclusion: Thus, OLO has similar pattern of degradation profiling in all hydrolytic conditions (acidic, alkaline and neutral). No degradation was found in thermal, UV light and oxidative conditions for ten days. OLO-Imp was recognized as analogue structure of OLO and proposed as 11 - [(3-dimethylamino)- propylidene] - 6, 11-dihydro-dibenz[b,e]oxepin-2-propanoic acid in standard drug. OLO1 was identified as (2-(4-(dimethylamino)butyl) phenyl)methanol which may be formed by cleavage of tricyclic ring in neutral condition.

Keywords: Olopatadine hydrochloride, Forced degradation, HPLC

INTRODUCTION

Forced degradation is a degradation of new drug substance or drug product at more severe conditions than accelerated conditions. Forced degradation studies afford the information about identification of possible degradants; degradation pathways and inherent stability of the active pharmaceutical ingredient. It also offers the information about any possible polymorphic or enantiomeric substances and difference between drug related degradation and excipient interferences. New Drug Application (NDA) registration requires data of forced degradation studies in the form of forced degradation products, degradation reaction kinetics, structure, mass balance, drug peak purity, etc. Therefore, forced degradation and impurity profiling is one of the input for NDA registration manuscript [1]. Due to the presence of impurities or degradation products USFDA has recalled the finished pharmaceutical products; few of them are as: adagen (pegademase bovine) injection, azelastin hydrochloride ophthalmic solution, brimonidine tartrate ophthalmic solution, cyclopirox gel, diflorasone diacetate cream, fludeoxyglucose F18 injections, hydroxyzine hydrochloride oral solution, leflunomide tablets, pediatric atropine sulfate injection, prednisolone sodium phosphate oral solution, ropinirole hydrochloride tablets and topiramate tablets [2]. Intrinsic stability of armodafinil hydrochloride was explored by forced degradation and impurity profiling, where structure of four degradation products were established and possible pathway was postulated. In the present work, whole research methodology was adopted from the Jain *et al* [3].

Chemically, olopatadine (OLO) is 11-((*Z*)-3-(dimethyl-amino) propylidene)-6,11-dihydro-dibenz[b,e] oxepin-2-acetic acid [4] and indicated for allergic rhinitis, urticaria, itching resulting from skin diseases such as eczema/dermatitis, prurigo, pruritus cutaneous, psoriasis vulgaris, multiform exudative erythema. OLO primarily acts as a selective histamine H1 receptor antagonist. It also inhibits production and release of chemical mediators such as leukotriene and thromboxane; the release of the neurotransmitter tachykinin [5–9]. Although, analytical methods for OLO determination in different matrix have been reported viz. radioimmunoassay [10], LC with tandem mass spectrometry [11], LC–ESI-MS–MS [12-13], capillary zone electrophoretic method [14], HPLC and HPTLC [15]; but there is on study is reported on forced degradation profiling including intrinsic stability of OLO. The objective of forced degradation is to provide guidance for developing of executing purposeful degradation experiments for pharmaceutical

drug candidates. The ICH Guideline provides some guidance on stress testing or purposeful degradation stress testing helps to determine the intrinsic stability of the molecule by establishing degradation pathways in order to identify the likely degradation products [16]. The objective of present research work was to establish intrinsic stability and forced degradation profiling of OLO by reverse phase high performance liquid chromatography diode array detector high-resolution mass spectrometry (RP-HPLC-DAD-HRMS) technique.

EXPERIMENTAL

Chemicals and reagent

Working standard of olopatadine hydrochloride (OLO) was gifted by Ranbaxy Laboratories Limited, Gurgaon, Harayana. HPLC grade solvents (methanol and acetonitrile), formic acid, hydrochloric acid, sodium hydroxide and hydrogen peroxide were procured from Merck Specialties Private Limited Mumbai, India. Triple distilled water was prepared in-house.

Instrument and chromatography

All dilutions, mobile phase and other solutions were filter through 0.2 µ nylon filter and chromatographed by Agilent Infinity 1260 series equipped with 1260 binary pump VL 400 bar, 1260 manual injector 600 bar, Rheodyne 7725i 7-port sample injection valve with 20 µL fixed loop, ZORBAX Eclipse Plus C18 (250 × 4.6 mm, 5 µm), 1260 DAD VL, 20 Hz detector, standard flow cell 10 mm, 13 µL, 120 bar, OpenLab CDS EZChrom Ed. Workstation and syringe 50.0 µL, FN, LC tip. Micromass Q-TOF micro (Waters) liquid chromatography-mass spectrometry (LC-MS) was performed in high resolution mass spectrometry (HRMS) mode by using electro spray positive ionization. The parameters viz. desolvation gas (500 Lt/hr), cone gas (25 Lt/hr), desolvation temperature (250 °C), source temperature (120 °C), capillary voltage (3000 V), cone voltage (30 V) and collision energy (10 V) were used for HRMS analysis. All dilutions, mobile phase and other solutions were used for the analysis were filtered through 0.2 µ nylon filter (Ultipor®N66 Nylon 6,6 membrane, Pall Sciences, Pall India Pvt. Ltd. Mumbai, India). The mobile phase composed of water adjusted to 0.1% formic acid (A) and acetonitrile and methanol (50:50) (B) in a gradient mode (Tmin/A:B; T0/70:30; T4/70:30; T14/20:80;

T22/80:20; T25/70:30). The flow rate was set to 1.0 mL/min with UV detector wavelength fixed at 300 nm. The injection volume was 20 µL.

Standard solution

Accurately weighed amount of 100 mg of OLO was dissolved in 100 mL of methanol to prepare stock P (1000 µg/mL). Aliquot of the stock P was diluted to prepare stock Q (100 µg/mL), which was used to prepare series of standard dilutions viz. 2, 4, 6, 8 and 10 µg/mL of OLO.

Method Validation

As per ICH guidelines [17-19], the proposed method was validated for the stability indicating assay and to establish inherent stability of OLO drug with forced degradation profiling in different conditions by RP-HPLC determination. The developed method was validated to assure the reliability of results of analysis for different parameters viz. linearity, range, accuracy, precision, robustness, limit of quantification (LOQ), limit of detection (LOD) and specificity. The serial dilutions (2-10 µg/ml) of the OLO in 50% methanol in triplicates were used for the linearity determination. Accuracy was determined by recovery method by spiking the standard solution to the pre-analyzed samples. Precision of the method was studied under the head of repeatability and intermediate precision. The six replicates of 10 µg/ml were chromatographed subsequently to assure the repeatability. The intermediate precision was determined by day to day analysis variation and analyst to analyst variation in the linearity range. The robustness of the method was studied with the variation in the temperature (20, 25 and 30°C) and content of formic acid (0.1%) in aqueous phase variation by 5% change. Limit of detection (LOD) and limit of quantification (LOQ) were determined by signal-to-noise ratio. The specificity was ascertained by degrading the drug sample in stressed conditions. Sample solution stability was demonstrated by analyzing six replicates of 10 µg/mL OLO standard samples at different time intervals with freshly prepared mobile phase on each time.

Forced degradation

Forced degradation studies of bulk drug included appropriate solid state (thermal and photolytic) and solution state stress conditions (hydrolytic and oxidative). The stock solution was used for the forced degradation study to provide an indication of the stability indicating property and

specificity of proposed method. Accurately weighed about 100 mg OLO was dissolved in 50 mL of methanol and volume was made upto 100 mL with water/ 0.2 N HCl / 0.2 N NaOH to perform hydrolytic degradation in neutral (water), acidic (0.1 N HCl) and alkali (0.1 N NaOH) conditions, respectively and refluxed at 60°C. Bulk drug (powder form) was exposed to 60 °C (hot air oven) and UV light separately for temperature and ultra-violet degradation, respectively. Oxidative degradation of OLO was performed in 3% and 10% H_2O_2 solution. Prior to injection, samples were withdrawn at appropriate time, neutralized (in case of acid and alkali hydrolysis) and the solutions were diluted with 50% methanol. Thermal and photolytic degraded drug (solid powder) was dissolved in 50% methanol to prepare sample for injection.

RESULT AND DISCUSSION

Optimization of chromatographic conditions

Although, OLO was eluted at 3.77 minute by the mixture of 0.1% formic acid and methanol (35:65) on Zorbax Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm) at 1.0 mL/min flow rate and it was successfully applied for determination of the OLO drug in dosage form [20] but it was not suitable for the separation of the degradation products along with OLO.

Thus, chromatographic conditions were optimized to develop a stability-indicating assay method to separate the related compounds and degradation products from the drug content. RP-HPLC mode chromatography was selected based on the solubility and pKa value of OLO. ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) was used separate all degradants along with drug. The wavelength of 300 nm was selected due to maximum absorbance of all the degradation product and drug, which was optimized by 1260 DAD VL, 20 Hz detector.

Broadening and splitting of peak were observed with the both mobile phases methanol and water (50:50) and acetonitrile and water (50:50). Drug's peak was merged with diluent as organic phase was increased. After using acidic aqueous phase (pKa of drug 4.29); the broadening and tailing of OLO peak was persisted. It was improved by increasing organic phase (methanol) upto 65%. Further, methanol content was reduced to 60% to resolve grouped 4-5 peaks in degraded samples; where little separation was found but peak shapes were not acceptable. Then, acetonitrile content was incorporated in organic phase along with methanol (50:50). All peaks of degraded samples were found very sharp but not well resolved with 0.1% formic acid and above

organic phase (40:60). Degradant peaks were well separated till 6 min when this ratio was reversed (60:40); but highly tailing was observed in drug's peak. Thus, degradants were well resolved in 60:40 ratios while drug eluted in 40:60; then it was concluded to run gradient mode.

Degradation products were resolved before drug peak but few of them were merged with dug peak in between 11-13 min and two degradation products were merged to each other at 16.5 min in the gradient mode (Tmin/A:B; T0/70:30; T7/70:30; T9/30:70; T17/70:30; T20/70:30). Basically, above elution mode is not purely gradient but it was combination of more isocratic and less gradient mode. Fine resolution was observed till 16 min, when gradient mode was increased (Tmin/A:B; T0/70:30; T5/70:30; T16/30:70; T17/70:30; T20/70:30), but group of degradation product were observed in between 16-19 min. So, the run time was extended to 25 min with increased gradient mode (Tmin/A:B; T0/70:30; T4/70:30; T20/30:70; T22/70:30; T25/70:30). Most of degradation products were resolved but tailing in drug peak was seen which may be due to less exposure to the organic phase, so it was decided to increase organic content in mobile phase (Tmin/A:B; T0/70:30; T4/70:30; T14/20:80; T22/70:30; T25/70:30). In this gradient mode, OLO and all degradation products in different forced degradation conditions were well separated with acceptable resolution.

Method validation

The consistence performance of HPLC system was assured by system suitability parameters. As per section 4.5.4.1, six replicates of OLO samples were injected, and column performances like tailing factors, retention time and number of theoretical plates were observed. The values of % relative standard deviation (% RSD) for these parameters were found within the acceptance criteria of system performance. OLO has better separation in set of conditions as the higher theoretical plates (59526) with less than one unit of % RSD (0.88). The tailing factor was found to be 0.68 with acceptable % RSD. The capacity factor was 15.02 i.e., OLO has sufficient opportunity to interact with the stationary phase resulting in differential migrations. The co-elution of impurities or degradants is generally investigated by examining the peak purity using a PDA detector. Three-dimensional view of the chromatogram has been confirmed that there was no peak around the OLO elution time. Here, the peak purity at 11.62 min was 1.0; there was no

interference with elution of OLO at retention time, or nothing was co-eluting along with OLO at 11.62 min. Thus, all system suitability parameters were found within the acceptance criteria.

The linear regression equation was found to be Y = 16751x + 26.50 with correlation coefficient r^2 = 0.999 and calibration graph was plotted for concentration versus area found in chromatogram. The accuracy of the developed method was assured at all levels of linearity. It was found in between 99.50 – 100.28% by recovery method with % RSD of 0.32; which shows the reliability for accuracy of the developed RP-HPLC method. The developed method was performed under two heads: repeatability and intermediate precision. The repeatability of sample injections was measured as determined amount of OLO in different serial six replicates which was expressed 99.89% with % RSD of 0.08, while intermediate precision was evaluated on the heads of inter-day analysis as well as analyst-to-analyst. Both parameters were determined near to 100% (99.93% and 99.95%) with less than unit % RSD (0.11 and 0.12) at all three levels of concentration.

The robustness of developed method was assured by the variation in amount of formic acid (0.1%) in aqueous phase of mobile phase composition and temperature of real time analysis. The effect of three variations in formic acid in aqueous phase (0.105%, 0.100% and 0.095%) and temperature variation (20, 25 and 30 °C) were observed no significant effect of change in parameters on results. These were found to be 99.97 and 100.17, with % RSD 0.13 and 0.29, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were determined by signal-to-noise ratio. These were found to be 0.045 µg/mL and 0.149 µg/mL, which show the sensitivity of the developed method. The % RSD value was less than two. Analysis of degraded drug sample in stressed conditions was evidence for specificity of the method. The retention time, peak symmetry, peak purity and UV spectrum of the recorded chromatogram different samples were confirmed to assure the specificity. The AUC of OLO peak in chromatogram has been decreased with increase in the AUC of degradant's peak. This experimental evidence confirmed that the developed method was specific for the analysis of OLO. The stability of OLO was assured during analysis by response ratio. The response ratio of the drug at all concentration level was varied in between 16565.67-16973.33 with % RSD of 0.89. It is evidence that the drug is stable in selected solution at room temperature and RP-HPLC method is continuously sensitive toward the analyte.

Forced degradation profiling

Acidic degradation: Five degradation products (DPs) viz. OLO1 (5.17), OLO2 (7.87), OLO3 (11.11), OLO4 (12.65) and OLO5 (12.91) were observed in acidic condition (Fig 1). OLO5 was major DP which was increased with time and peak area of OLO was decreased. All DPs were appeared on first day except OLO1 and OLO2 which were appeared on fourth and second day, respectively. Peak area of OLO3 and OLO4 was increased first and then decreased. Other two DPs OLO1 and OLO2 increased with time.

Alkaline degradation: OLO was gradually degraded into four DPs viz. OLO3 (11.11), OLO6 (12.07), OLO5 (12.91) and OLO7 (17.48) in alkaline condition (Fig 2). OLO3 and OLO5 were common DPs in acidic and alkaline conditions while OLO6 and OLO7 were newer DPs in basic degradation. OLO5 and OLO6 were two major DPs; OLO5 was increased with time while OLO6 has zig-zag pattern of peak area with time. Concentration of both OLO3 and OLO7 were increased with time.

Neutral degradation: All DPs of neutral condition were also found in acidic condition while OLO3 and OLO5 were common in all three type of hydrolytic degradation (Fig 3). OLO5 was major DP in all conditions. OLO was gradually degraded and peak area of all DPs increased with time in neutral conditions. RP-HPLC analysis of stressed samples of OLO in thermal, oxidative and photolytic degradation has shown that there was no degradation in these conditions. It might be possible that due to solid sample in thermal stress condition no degradation was observed.

Thus, in acidic condition, five degradation products (DPs) viz. OLO1, OLO2, OLO3, OLO4 and OLO5 were observed. OLO5 was major DP which was increased with time and peak area of OLO was decreased. In addition to OLO3 and OLO5; two more DPs were observed in alkaline condition viz. OLO6, and OLO7 in alkaline condition. OLO5 and OLO6 were two major DPs; OLO5 was increased with time while OLO6 has zig-zag pattern of peak area with time. All DPs of neutral condition were also found in acidic condition while OLO3 and OLO5 were common in all three type of hydrolytic degradation. Thus, OLO has similar pattern of degradation profiling in all hydrolytic conditions (acidic, alkaline and neutral) (Fig 4). No degradation was found in thermal and UV light exposure, as these were performed on solid powder of OLO, so it might be

possible that degradation is less prone in the solid powder. Similarly, oxidative degradation was also not observed for ten days.

Degradation pathway and impurity profiling

Standard drug OLO: LC-MS study was performed in positive mode using leucine enkephalin as standard, where OLO and its impurity (OLO-Imp) were observed at RRT of 1.00 and 1.11, respectively. The ESI mass spectrum of OLO showed protonated molecular ion $[M+H]^+$ and its isotopic abundance $[M+2H]^+$ at m/z 338.1671 and 339.1663 with ppm errors in mass 2.08 and 4.45, respectively. It was advocated by its further fragmentation pattern into its daughter products as: 337.1678 → 292.1099 → 221.0961 → 247.1117 → 165.0546 (Table 1, Fig. 5).

An impurity OLO-Imp (OLO5) was observed in ESI spectrum, and its protonated molecular ion $[M+H]^+$ was observed at m/z 352.1840 along with its isotopic abundance (Fig. 6). It's structure was analogue of OLO and proposed as 11 - [(3 - dimethylamino)- propylidene] - 6, 11-dihydro-dibenz[b,e]oxepin-2-propanoic acid (Fig. 6). It was also used as internal standard for metabolite study [10].

Acidic degradation: In addition to OLO, sodium salt of OLO (OLO3) and OLO5 (OLO-Imp) were observed in MS spectrum of acidic condition sample at RRT of 0.96 and 1.11. OLO3 was observed in all three degradation conditions. Its protonated molecular ion $[M+H]^+$ was observed at m/z 360.1502, which has shown its carboxylic acid analogue at m/z 338.1684. The MS-MS fragmentation pattern into its daughter products advocated the purposed structure as: 359.1497 → 337.1678 → 279.1623 → 157.0891 (Table 2). OLO was also characterized in acidic degradation at RRT of 1.00. MS-ES^+ scanning has revealed protonated molecular ion $[M+H]^+$ of OLO at m/z 338.1676 with isotopic abundance. MS-MS of OLO in acidic condition has shown fragmentation pattern in mass spectrum as: 337.1678 → 292.1099 → 247.1117; 165.0546 (Table 2). The protonated molecular ion $[M+H]^+$ of OLO5 was observed at m/z 352.1821 with isotopic abundance at 353.1839 (Fig 7). The ppm error in mass was less than five units (Table 2).

Alkaline degradation: MS spectrum of alkaline sample of OLO was observed as similar to MS spectrum of acidic conditions. The degradation pathway and fragmentation pattern of OLO and its degradation products were as similar to acidic conditions (Fig. 8). Only ppm error in mass varies from acidic condition (Table 3).

Neutral degradation: In neutral condition, tricyclic ring has been cleavaged and OLO1 was formed (RRT = 0.44). OLO3 was also formed as similar pattern to acidic and alkaline conditions (RRT = 0.96). OLO5 (OLO-Imp) was also observed in MS spectrum as similar to all other conditions. Only one additional degradation product (OLO1) was observed in neutral condition, whose protonated molecular ion $[M+H]^+$ was found m/z at 208.1615 and identified as (2-(4-(dimethylamino)butyl) phenyl)methanol (Fig. 9). The proposed structure was further advocated by its degradation pattern (207.1623 → 177.1154 → 157.0891) (Table 4). OLO3, OLO and OLO5 were also characterized in neutral condition as similar to above both acidic and alkaline conditions. The ppm error in mass is different from above conditions but most of the error is below 5 units (Table 4).

Thus, OLO and its impurity (OLO-Imp) were observed at RRT of 1.00 and 1.11, respectively in LC-MS study. OLO-Imp was recognized as analogue structure of OLO and proposed as 11 - [(3-dimethylamino)- propylidene] - 6, 11-dihydro-dibenz[b,e]oxepin-2-propanoic acid (Fig 10). In addition to OLO, sodium salt of OLO (OLO3) and OLO5 (OLO-Imp) were observed in both MS spectrum of acidic and alkaline conditions sample at RRT of 0.96 and 1.11. OLO3 was observed in all three degradation conditions and identified as sodium salt of OLO (OLO is carboxylic acid derivative). Additionally, OLO1 (RRT = 0.44) was identified as (2-(4-(dimethylamino)butyl) phenyl)methanol which may be formed by cleavage of tricyclic ring in neutral condition. OLO is acetic acid derivative along with oxepin ring and tertiary nitrogen, which is quite stable form of drug. There is no vulnerable functional group which liable to convert or breakdown into degradation products under stressed or forced degradation conditions. So, OLO forms relatively slow and less number of degradation products.

CONCLUSION

OLO has similar pattern of degradation profiling in all hydrolytic conditions (acidic, alkaline and neutral), while no degradation was found in thermal, UV light and oxidative conditions for ten days. OLO-Imp was recognized as analogue structure of OLO and proposed as 11 - [(3-dimethylamino)- propylidene] - 6, 11-dihydro-dibenz[b,e]oxepin-2-propanoic acid. OLO3 was observed in all three degradation conditions and identified as sodium salt of OLO (OLO is

carboxylic acid derivative). Additionally, OLO1 was identified as (2-(4-(dimethylamino)butyl) phenyl)methanol which may be formed by cleavage of tricyclic ring in neutral condition.

ACKNOWLEDGEMENTS

One of the authors, Pawan Kumar Basniwal, is earnestly indebted to the Science and Engineering Research Board (SERB), DST, New Delhi, for the financial support for this research work under Fast Track Scheme for Young Scientists [SR/FT/LS-187/2008 (G)].

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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Table 1. Observed m/z values for $[M+H]^+$ ions and major fragments of OLO and its impurity

Analyte Code	RRT in LC MS	MS-ES or MS-MS	m/z value	Measured Mass	Best possible molecular formulae	Exact Mass	PPM error
OLO	1.00	MS-ES	338.1671	337.1671	$C_{21}H_{23}NO_3$	337.1678	2.08
			339.1663	337.1663	$C_{21}H_{23}NO_3$	337.1678	4.45
			340.1701	337.1701	$C_{21}H_{23}NO_3$	337.1678	-6.82
	1.00	MS-MS	338.1661	337.1661	$C_{21}H_{23}NO_3$	337.1678	5.04
			339.1667	337.1667	$C_{21}H_{23}NO_3$	337.1678	3.26
			293.1109	292.1109	$C_{19}H_{16}O_3$	292.1099	-3.42
			247.1098	247.1098	$C_{18}H_{15}O^+$	247.1117	7.69
			221.0982	221.0982	$C_{16}H_{13}O^+$	221.0961	-9.50
			165.0556	165.0556	$C_9H_9O_3^+$	165.0546	-6.06
OLO-Imp	1.11	MS-ES	352.1840	351.1840	$C_{22}H_{25}NO_3$	351.1834	-1.71
			353.1835	351.1835	$C_{22}H_{25}NO_3$	351.1834	-0.28

Table 2. Observed m/z values for the $[M+H]^+$ ions and major fragments of OLO and its degradation products in acidic condition

Analyte Code	RRT in LC MS	MS-ES or MS-MS	m/z value	Measured Mass	Best possible molecular formulae	Exact Mass	PPM error
OLO3	0.96	MS-ES	360.1502	359.1502	$C_{21}H_{22}NNaO_3$	359.1497	-1.39
			338.1684	337.1684	$C_{21}H_{23}NO_3$	337.1678	-1.78
	0.96	MS-MS	360.1491	359.1491	$C_{21}H_{22}NNaO_3$	359.1497	1.67
			361.1488	359.1488	$C_{21}H_{23}NO_3$	359.1497	2.51
			338.1676	337.1676	$C_{21}H_{23}NO_3$	337.1678	0.59
			280.1625	279.1625	$C_{19}H_{21}NO$	279.1623	-0.72
			158.0883	157.0883	$C_{11}H_{11}N$	157.0891	5.09
OLO	1.00	MS-ES	338.1676	337.1676	$C_{21}H_{23}NO_3$	337.1678	0.59
			339.1698	337.1698	$C_{21}H_{23}NO_3$	337.1678	-5.93
	1.00	MS-MS	338.1695	337.1695	$C_{21}H_{23}NO_3$	337.1678	-5.04
			339.1688	337.1688	$C_{21}H_{23}NO_3$	337.1678	-2.97
			293.1102	292.1102	$C_{19}H_{16}O_3$	292.1099	-1.03
			247.1132	247.1132	$C_{18}H_{15}O^+$	247.1117	-6.07
			165.0552	165.0552	$C_9H_9O_3^+$	165.0546	-3.64
OLO5	1.11	MS-ES	352.1821	351.1821	$C_{22}H_{25}NO_3$	351.1834	3.70
			353.1839	351.1839	$C_{22}H_{25}NO_3$	351.1834	-1.42

Table 3. Observed m/z values for the $[M+H]^+$ ions and major fragments of OLO and its degradation products in alkaline condition

Analyte Code	RRT in LC MS	MS-ES or MS-MS	m/z value	Measured Mass	Best possible molecular formulae	Exact Mass	PPM error
OLO3	0.96	MS-ES	360.1507	359.1507	$C_{21}H_{22}NNaO_3$	359.1497	-2.78
			338.1686	337.1686	$C_{21}H_{23}NO_3$	337.1678	-2.37
	0.96	MS-MS	360.1498	359.1498	$C_{21}H_{22}NNaO_3$	359.1497	-0.28
			361.1499	359.1499	$C_{21}H_{23}NO_3$	359.1497	-0.56
			338.1672	337.1672	$C_{21}H_{23}NO_3$	337.1678	1.78
			280.1629	279.1629	$C_{19}H_{21}NO$	279.1623	-2.15
			158.0890	157.0890	$C_{11}H_{11}N$	157.0891	0.64
OLO	1.00	MS-ES	338.1696	337.1696	$C_{21}H_{23}NO_3$	337.1678	-5.34
			339.1688	337.1688	$C_{21}H_{23}NO_3$	337.1678	-2.97
			340.1675	337.1675	$C_{21}H_{23}NO_3$	337.1678	0.89
	1.00	MS-MS	338.1685	337.1685	$C_{21}H_{23}NO_3$	337.1678	-2.08
			339.1688	337.1688	$C_{21}H_{23}NO_3$	337.1678	-2.97
			293.1108	292.1108	$C_{19}H_{16}O_3$	292.1099	-3.08
			247.1110	247.1110	$C_{18}H_{15}O^+$	247.1117	2.83
			165.0540	165.0540	$C_9H_9O_3^+$	165.0546	3.64
OLO5	1.11	MS-ES	352.1828	351.1828	$C_{22}H_{25}NO_3$	351.1834	1.71
			353.1839	351.1839	$C_{22}H_{25}NO_3$	351.1834	-1.42

Table 4. Observed m/z values for the $[M+H]^+$ ions and major fragments of OLO and its degradation products in neutral condition

Analyte Code	RRT in LC MS	MS-ES or MS-MS	m/z value	Measured Mass	Best possible molecular formulae	Exact Mass	PPM error
OLO1	0.44	MS-ES	208.1615	207.1615	$C_{13}H_{21}NO$	207.1623	3.86
			178.1164	177.1164	$C_{11}H_{15}NO$	177.1154	-5.65
			158.0883	157.0883	$C_{11}H_{11}N$	157.0891	5.09
OLO3	0.96	MS-ES	360.1489	359.1489	$C_{21}H_{22}NNaO_3$	359.1497	2.23
			361.1498	359.1498	$C_{21}H_{23}NO_3$	359.1497	-0.28
			338.1666	337.1666	$C_{21}H_{23}NO_3$	337.1678	3.56
			280.1618	279.1618	$C_{19}H_{21}NO$	279.1623	1.79
			158.0888	157.0888	$C_{11}H_{11}N$	157.0891	1.91
OLO	1.00	MS-ES	338.1681	337.1681	$C_{21}H_{23}NO_3$	337.1678	-0.89
			339.1685	337.1685	$C_{21}H_{23}NO_3$	337.1678	-2.08
	1.00	MS-MS	338.1687	337.1687	$C_{21}H_{23}NO_3$	337.1678	-2.67
			339.1667	337.1667	$C_{21}H_{23}NO_3$	337.1678	3.26
			293.1118	292.1118	$C_{19}H_{16}O_3$	292.1099	-6.50
			247.1119	247.1119	$C_{18}H_{15}O^+$	247.1117	-0.81
			221.0965	221.0965	$C_{16}H_{13}O^+$	221.0961	-1.81
			165.0551	165.0551	$C_9H_9O_3^+$	165.0546	-3.03
OLO5	1.11	MS-ES	352.1856	351.1856	$C_{22}H_{25}NO_3$	351.1834	-6.26
			353.1825	351.1825	$C_{22}H_{25}NO_3$	351.1834	2.56

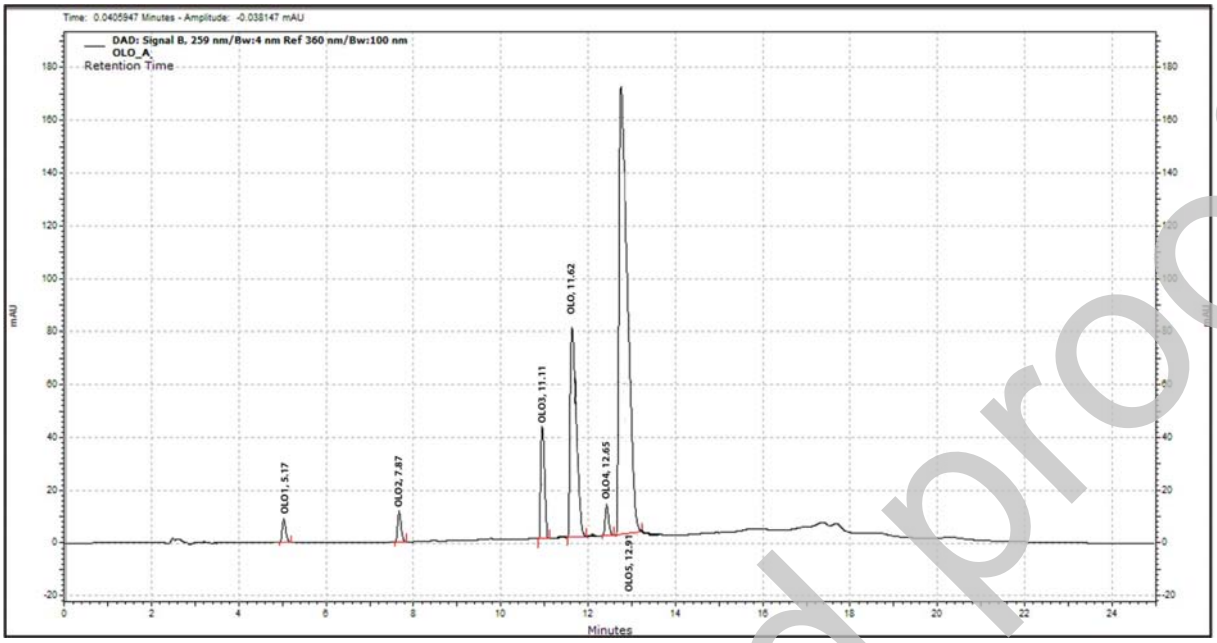

Figure 1. Representative chromatogram of OLO in acidic condition

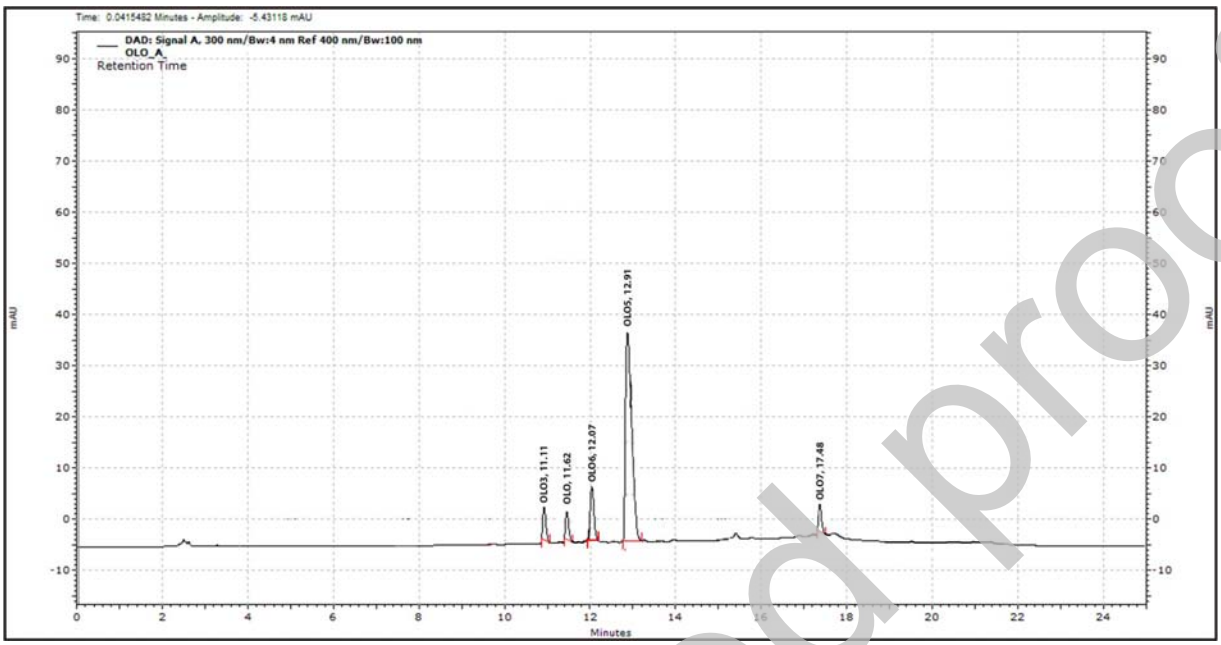

Figure 2. Representative chromatogram of OLO in alkaline condition

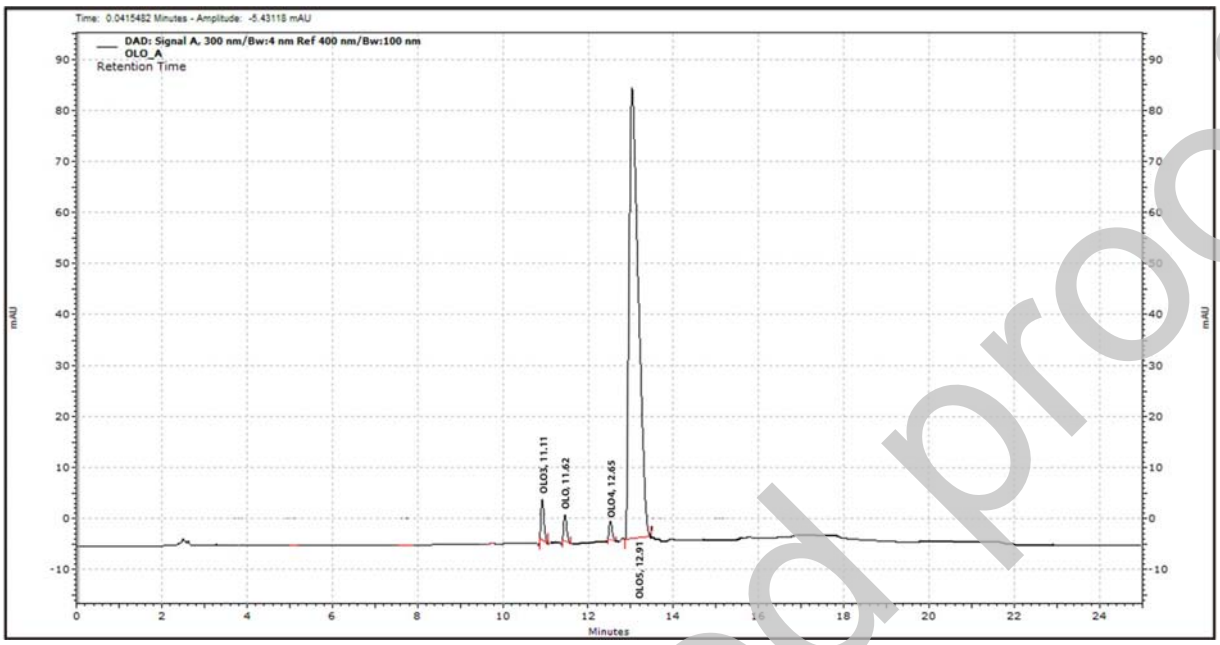

Figure 3. Representative chromatogram of OLO in neutral condition

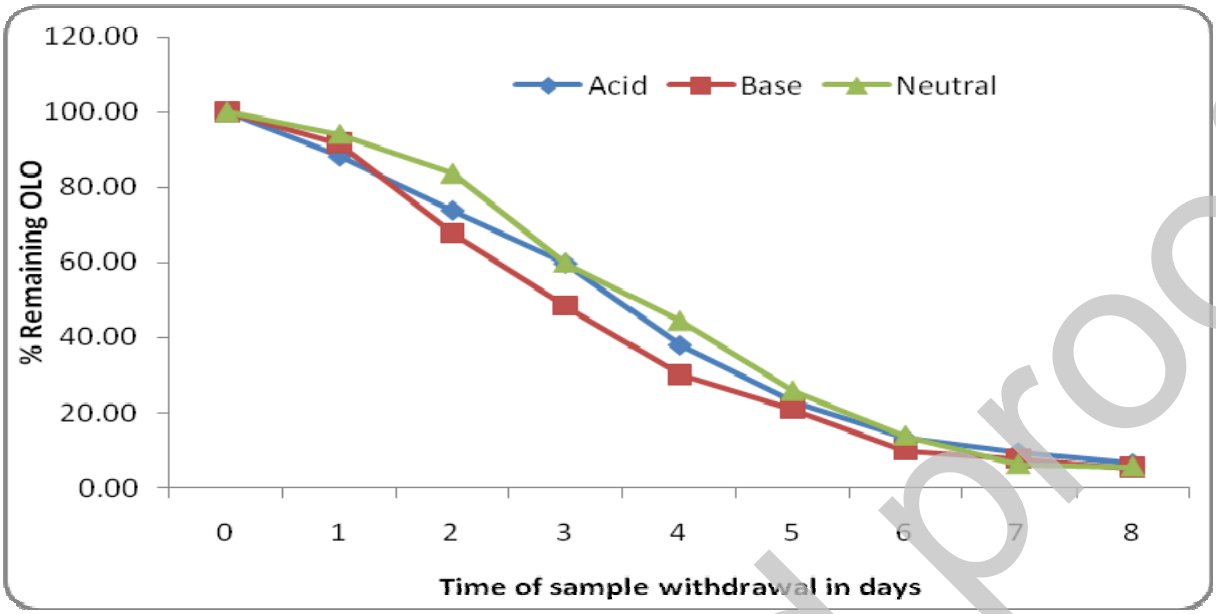

Figure 4. Degradation profiling of OLO in acidic, basic and neutral condition

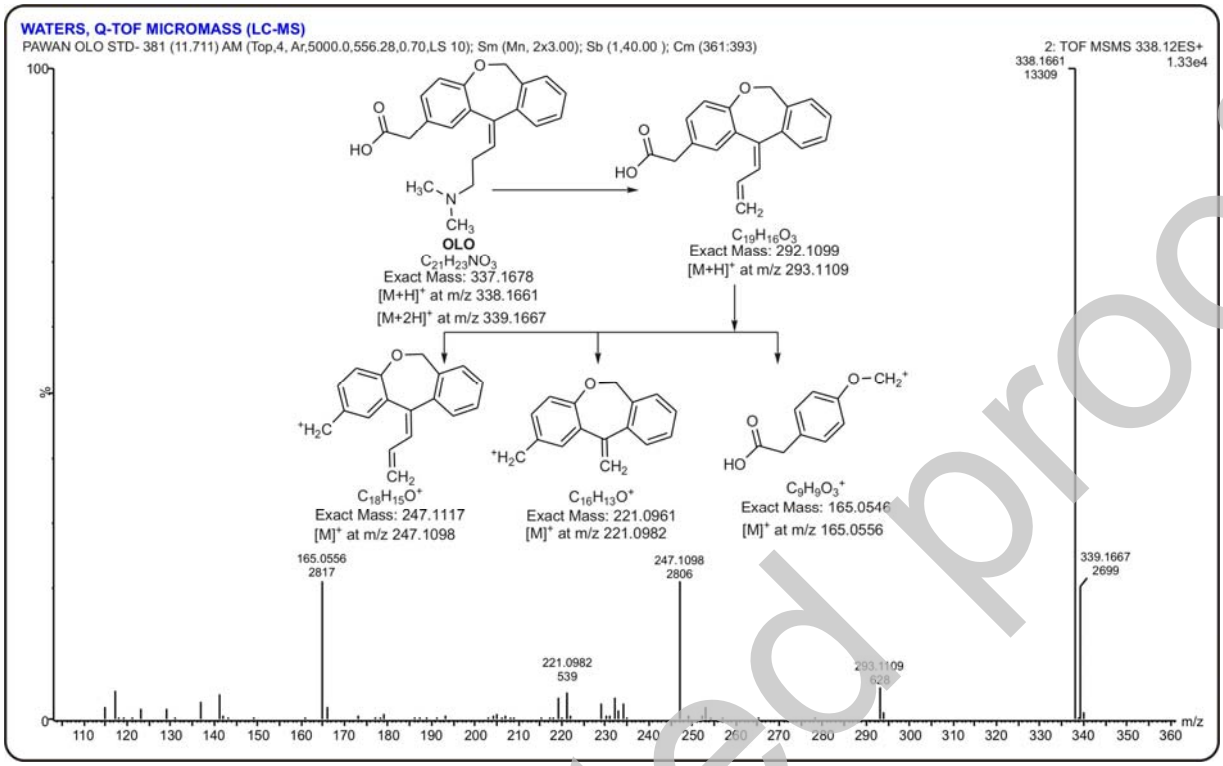

Figure 5. Chromatogram of OLO by TOF MS-MS spectrum and proposed fragmentation pattern of OLO

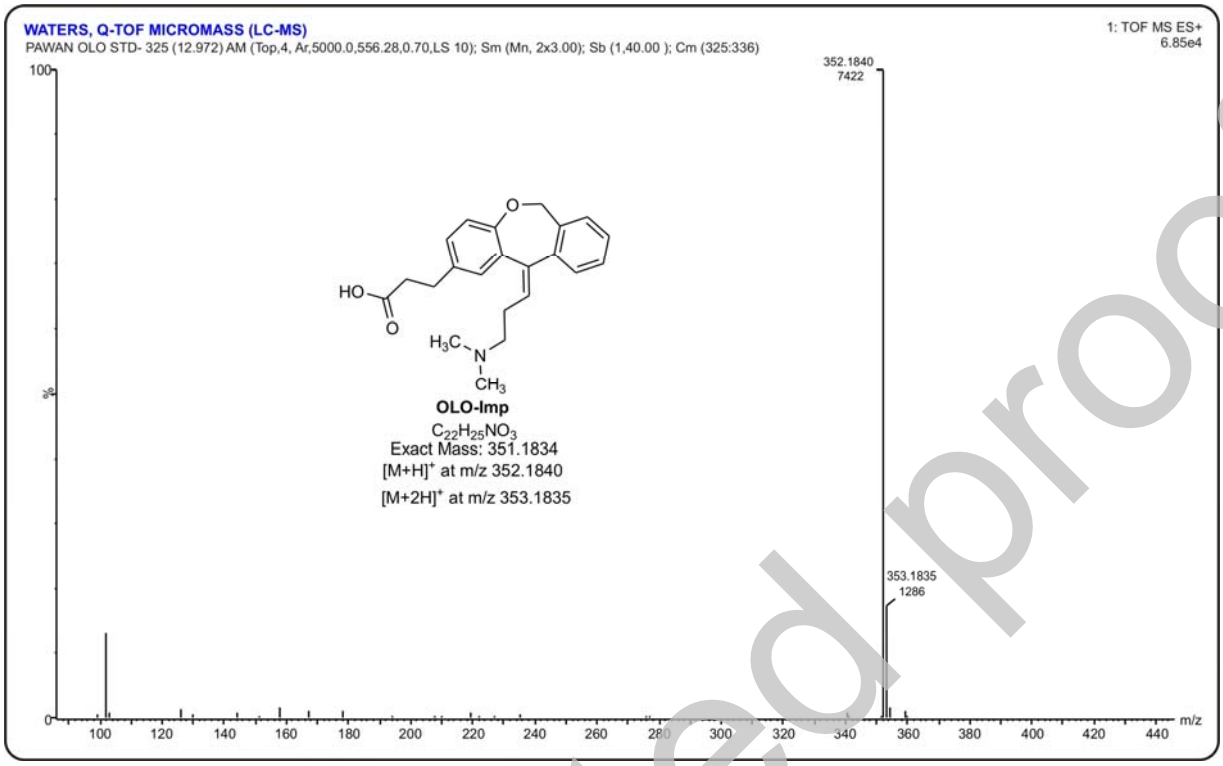

Figure 6. Chromatogram of OLO-Imp by TOF MS-ES$^+$ and proposed structure of OLO-Imp.

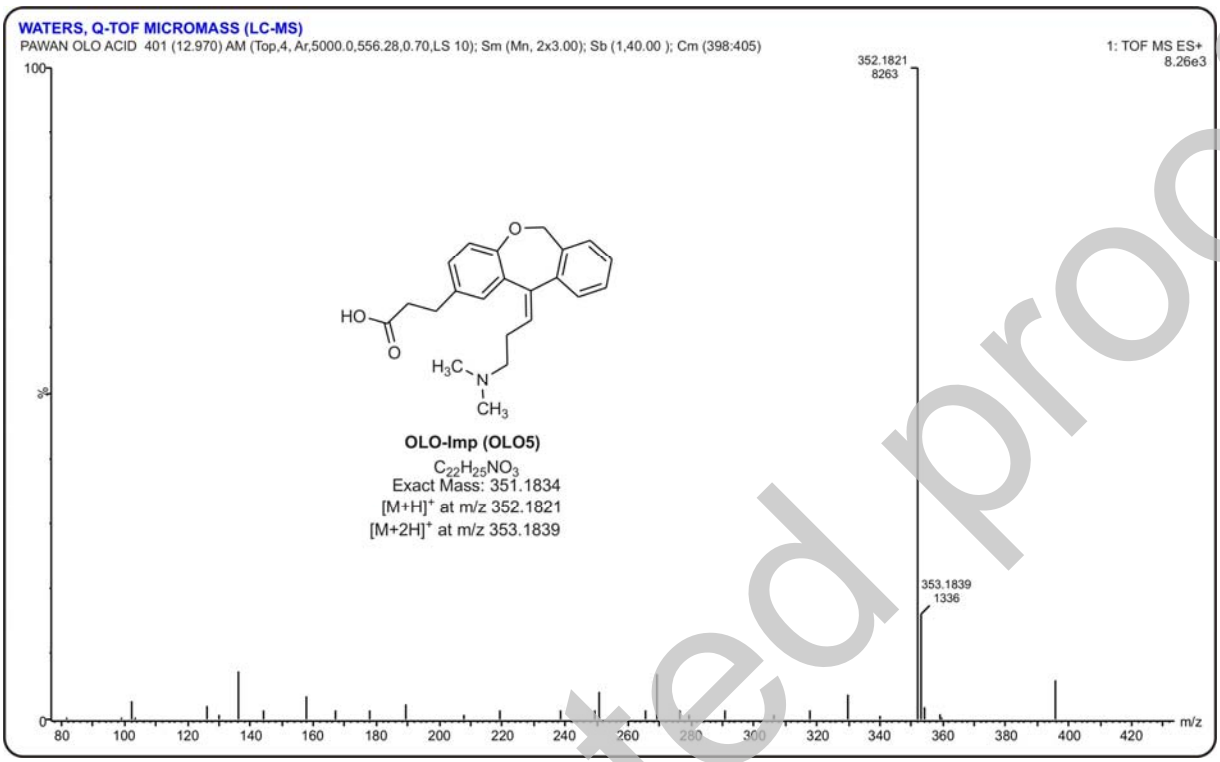

Figure 7. Chromatogram of OLO5 by TOF MS-ES+ spectrum and proposed structure of OLO5 in acidic condition

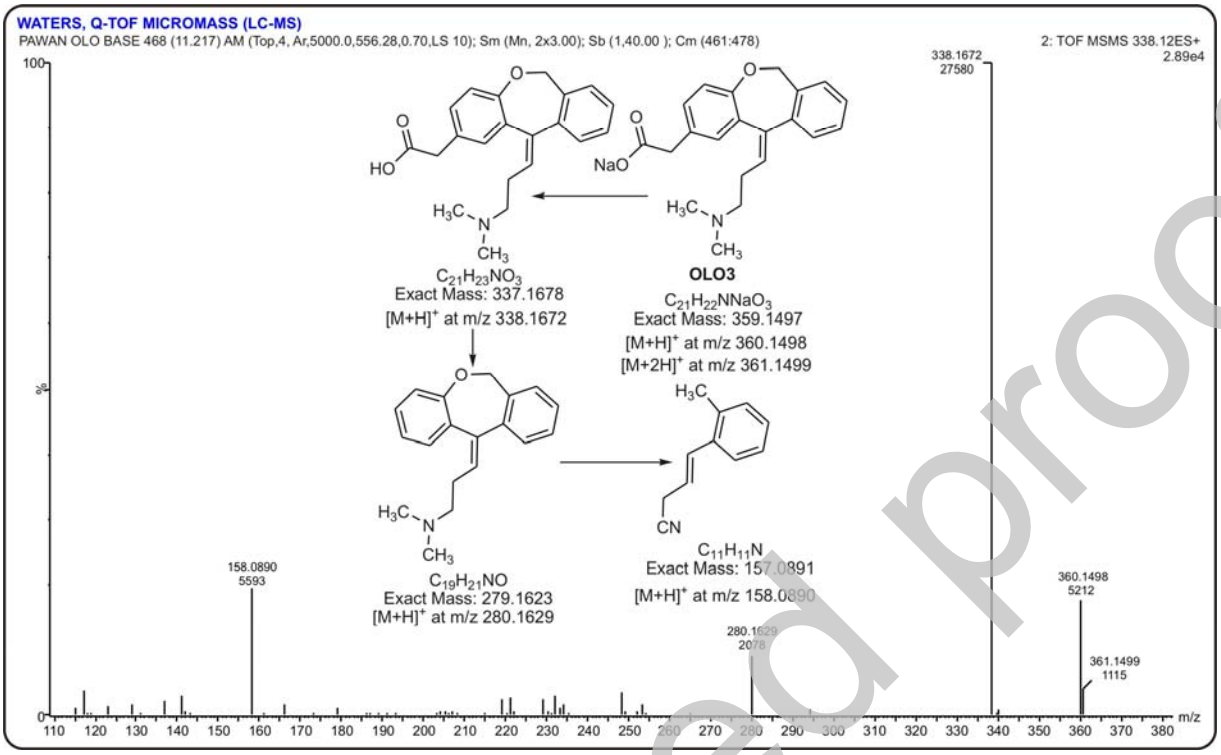

Figure 8. Chromatogram of degraded OLO in alkaline condition by TOF MS-MS spectrum and proposed fragmentation pattern of OLO3

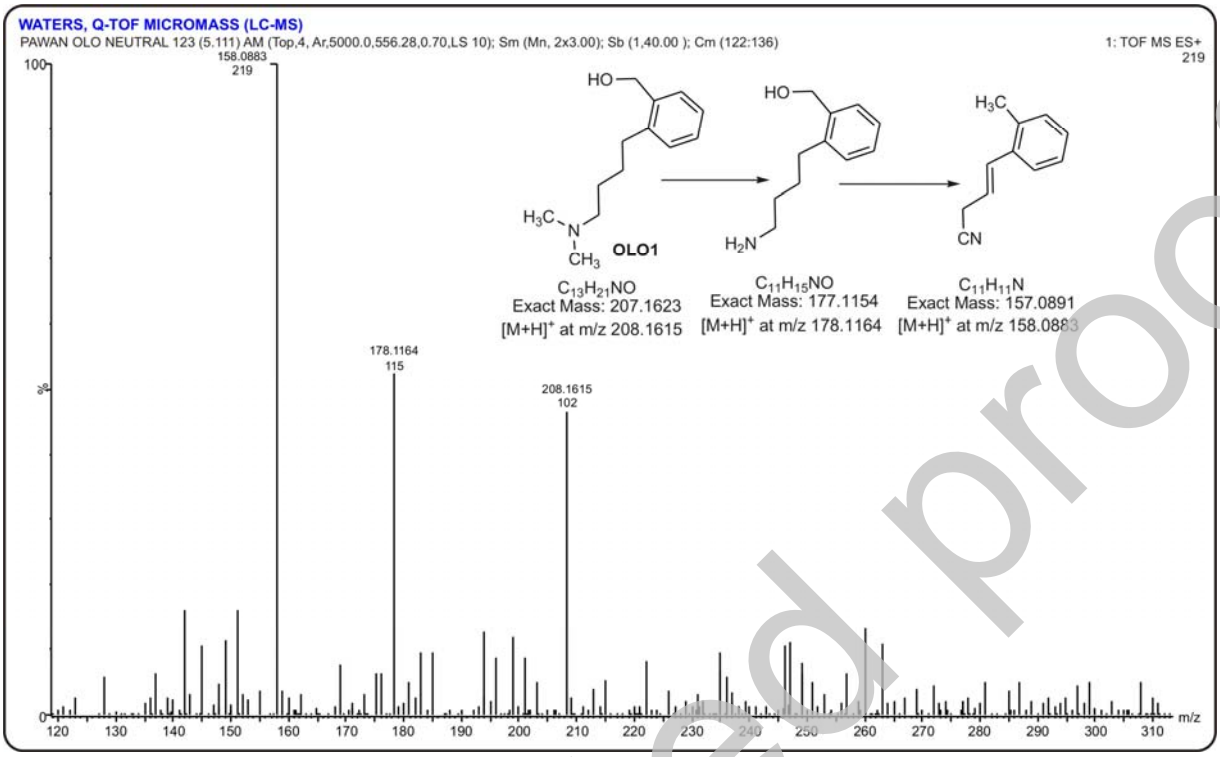

Figure 9. Chromatogram of degraded OLO in neutral condition by TOF MS-ES+ spectrum and proposed fragmentation pattern of OLO1

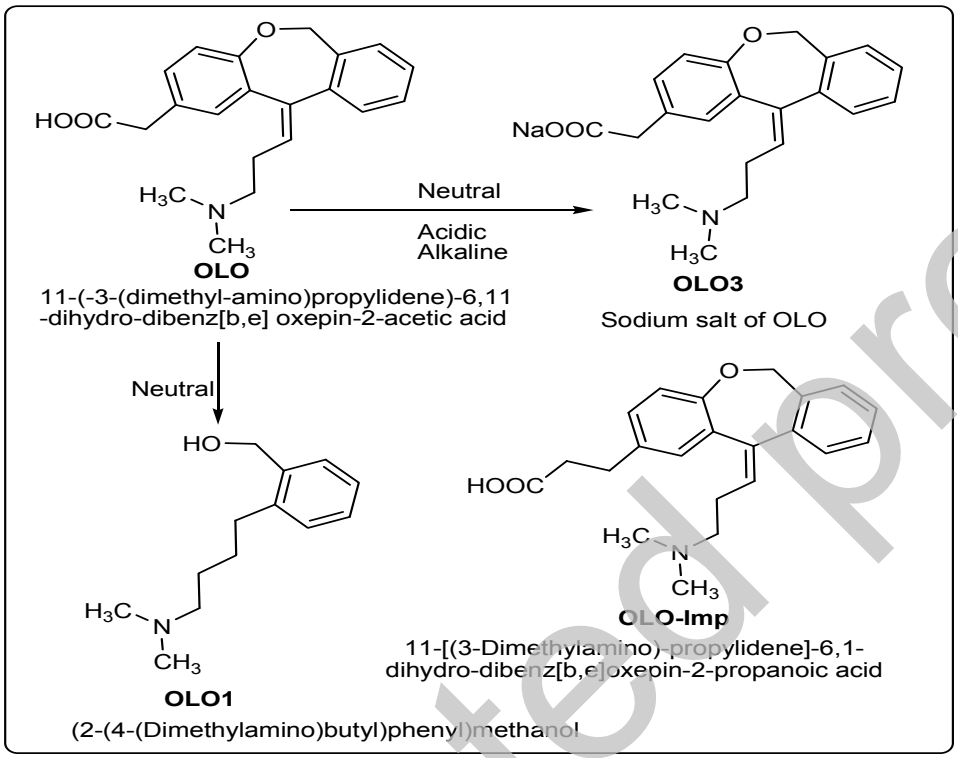

Figure 10. Proposed structure of OLO-Imp and proposed degradation pathway of OLO in acidic, alkaline and neutral condition