Screening and Identification of Potential Genotoxic Degradation Impurities using Q-TOF LC/MS with Advanced Software Solutions

Application Note

Pharmaceuticals

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Abstract

This Application Note demonstrates Agilent capabilities to screen, identify, and isolate potential genotoxic degradants using a combination of Agilent LC and LC/MS systems with various Agilent software solutions. This study used oxidative degraded atorvastatin calcium as the test substance. The Agilent 1290 Infinity LC System screened the extent of degradation and the Agilent 6530 Accurate Mass Q-TOF LC/MS System obtained MS and MS/MS information to identify impurities. The Agilent MassHunter Qualitative Analysis Software and the Agilent Molecular Structure Correlator (MSC) Software processed the data to propose possible structures for degradants. From the MSC proposed list, impurities having functional groups with indication of genotoxic potential were labeled as genotoxic impurities. The MS/MS fragmentation pattern of these genotoxic impurities were compared with known similar structures to confirm the findings. Fractions of the genotoxic impurities were isolated from complex degradation samples using the Agilent mass-based fraction collection system. This fractionation process can be scaled up to get a sufficient amount of the impurity for further structure confirmation, using techniques such as NMR.

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Introduction

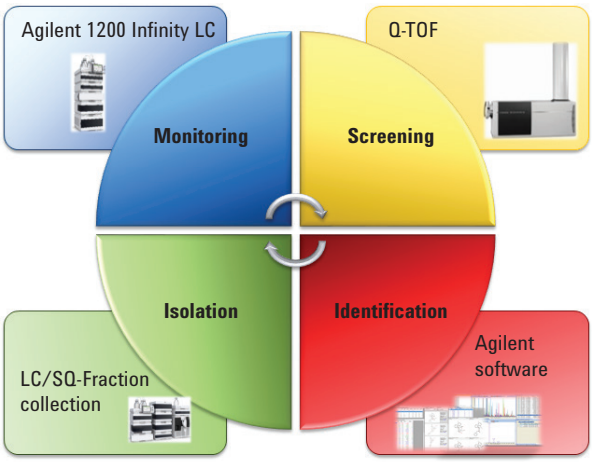
Drug substance purity is critical to ensure drug safety and quality. Many drugs are sensitive to environmental conditions and susceptible to degradation. It is standard practice to study the stability profile of a drug substance under predetermined stress conditions. Oxidative degradation of some drugs during handling or storage can lead to the formation of genotoxic impurities. Pharmaceutical manufacturers are required to monitor and identify genotoxic impurities at low levels.

In 2009, Matja *et al*. reviewed the oxidative degradation mechanism of atorvastatin and the formation of epoxide impurities[1]. Grahek *et al*. underlined these findings in 2011[2]. Epoxides are electrophilic due to a strained epoxide ring[3,4]. Many aliphatic and aromatic epoxides are mutagenic as a consequence of this chemical reactivity[5]. Peroxyl radicals can react to alkenes leading to the formation of hydroperoxides. The most prevalent analytical technique used for epoxide analysis is High Performance Liquid Chromatography (HPLC)[5]. This Application Note demonstrates a quick and reliable software-assisted solution for screening and identifying trace-level potential genotoxic epoxide degradants from atorvastatin using MS/MS data. The method demonstrates mass-based fraction collection of a genotoxic impurity for further characterization.

Experimental

Reagents and chemicals

Atorvastatin calcium drug substance was purchased from Aldrich. All solvents used for analysis were of LC/MS grade, and were purchased from Fluka. Buffers and reagents were purchased from Aldrich. Purified water was obtained from a Milli-Q water purification system (Millipore, USA).



Instrumentation

HPLC/UV analysis was performed using an Agilent 1290 Infinity Binary LC System consisting of:

- Agilent 1290 Infinity Binary Pump 4220A
- Agilent 1290 Infinity Autosampler G4226A
- Agilent 1290 Infinity Thermostat G1330B
- Agilent 1290 Infinity Thermostatted Column Compartment G1316C
- Agilent 1290 Infinity Diode Array Detector G4212A with 60-mm Max-Light flow cell (p/n G4212-60007)
- Operated with Agilent ChemStation OpenLab CDS Software (v. C.01.05). The chromatography parameters are given in Table 1.

Table 1. Agilent 1290 Infinity LC System chromatographic run conditions.

Buffer	3.9 g/L of ammonium acetate in water, pH 5.0 with glacial acetic acid	
Mobile phases	A) Acetonitrile, tetrahydrofuran, and buffer (21:12:67) B) Acetonitrile, tetrahydrofuran, and buffer (61:12:27)	
Column	Agilent Poroshell 120 EC-C8, 2.1 × 100 mm, 2.7 µm column at 30 °C	
Injection volume	1.5 µL	
Needle wash	50:50 methanol:water	
Flow rate	0.31 mL/min	
Gradient	Time (min)	Mobile phase B (%)
	0	0
	16	0
	28	80
	34	100
	40	100
	42	0
	46	0
DAD	Signal 244/4 nm, 60-mm flow cell, > 0.05 minutes (5 Hz)	

To screen impurities, an Agilent 1290 Infinity LC System was coupled to an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS with an Agilent Jet Steam (AJS) dual source. The Agilent MassHunter Workstation Software (B.05.01) acquired the data. Table 2 lists 6530 Q-TOF LC/MS acquisition and source parameters.

For impurity isolation, an Agilent 6150 Single Quadrupole LC/MS System and an Agilent 1260 Infinity Fraction Collector Bio-FC-AS (G5664A) module were connected to an Agilent 1290 Infinity Binary LC System and operated with Agilent ChemStation OpenLab CDS Software. The 1260 Infinity Fraction Collector run conditions are given in Table 3.

Procedure

Oxidative degradation of atorvastatin calcium was carried out by treating the drug substance with 3 % hydrogen peroxide at 40 °C for 24 hours. The functional group with labile hydrogen is susceptible to oxidation, and forms hydroperoxides, hydroxides, or ketones. The 1290 Infinity LC/UV System using the USP listed Atorvastatin liquid chromatography assay method monitored the extent of degradation[6,7]. To screen for impurities, samples with and without degradation (control) were analyzed using a 6530 Q-TOF LC/MS in both positive and negative ionization modes. MassHunter software identified impurities with potential genotoxicity, while a 6150 Single Quadrupole LC/MS coupled with a 1260 Infinity Fraction Collector isolated potential genotoxic impurities.

Table 2. Agilent 6530 Q-TOF LC/MS acquisition parameters.

Acquisition mode	MS and auto MS/MS
Ion polarity	Positive and negative in alternative runs
Gas temperature	275 °C
Gas flow	5 L/min
Nebulizer	45 psi
Sheath gas temperature	325 °C
Sheath gas flow	12 L/min
Nozzle voltage	500 V
Fragmentor	75 V
Skimmer	65 V
Collision energy	1 V
V Cap	4,000
MS, scan range	100–1,700 *m/z* at 1 spectra/s rate
MS/MS, scan range	50–1,700 *m/z* at 3 spectra/s rate
Isolation width MS/MS	Medium (4 amu)
Ramped collision energy	Slope 5, Offset 2.5

Table 3. Agilent 6150 Single Quadrupole LC/MS and Agilent Infinity 1260 Infinity Fraction Collector parameters.

Mode	Negative ionization in SIM and scan mode
Drying gas flow	8 L/min
Nebulizer Pressure	35 psi
Drying gas temperature	300 °C
Sheath gas temperature	250 °C
Sheath gas flow	5 L/min
Capillary cap	1,300 V
Nozzle voltage	2,000 V
Dwell time	195 msec
Collector delay	0.2 minutes
Fragmentor	70 V
Peak width	0.1 minutes
Fraction trigger mode	Peak-based
Max peak duration	0.5 minutes
Active split ratio	7:1
Frequency	2.222 (Hz)
Vol	300 µL
Injection volume	20 µL (needle wash for 10 seconds using acetonitrile:water 70:30)
Sample target mass	431.1, 465.1, 501.1
SIM ions	430.1, 464.1

Data analysis workflow

The Agilent MassHunter Profinder (B.06.00) extracted differential impurities between the nondegraded (control) samples and the degraded samples. Agilent Mass Profiler Professional (MPP) statistical analysis software (12.6.1) then analyzed the extracted impurities and generated a list of impurities with a fold change greater than two. ID Browser (MassHunter Qualitative Software Find by Formula algorithm) searched the list using the database developed by MassHunter PCDL manager (B.04.00). The database included accurate mass and formulas of known impurities and degradants of atorvastatin from literature and Mass-Meta-Site Software (4.1.1 Molecular Discovery Ltd).

The precursor and fragment ion information of unknown impurities (greater than two fold change) processed by the Agilent MassHunter Molecular Feature Extraction (MFE) algorithm was imported into Agilent Molecular Structure Correlator (MSC) software to propose possible structures. The details of the MSC software and multiple approaches to identify impurities can be found in Agilent publication 5991-1375EN[8]. Structure search in MSC includes the following steps:

1. Connect to ChemSpider.
2. Retrieve structures from ChemSpider for the proposed molecular formula.
3. Assign theoretical substructure on the experimental data.
4. Filter out all impurities with an overall score lower to set value and display all qualifying structures whose theoretical fragmentation matched to experimental fragmentation pattern.

Potential genotoxic impurities were identified and labelled from the list of proposed structures. Data analysis workflow using various software solutions are summarized in Figure 1.

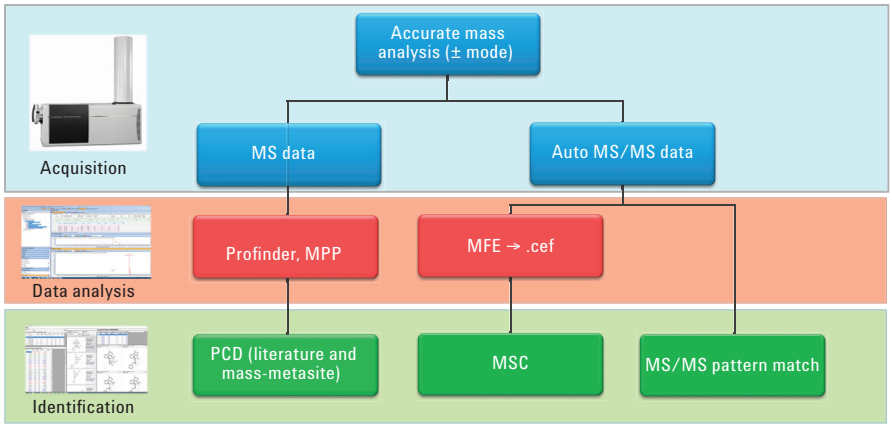


Figure 1. Software workflow which involves various Agilent software tools to identify known and unknown impurities.

Results and Discussion

Monitoring extend of degradation

Figure 2 illustrates HPLC/UV data of the control and degraded atorvastatin samples analyzed using the modified USP method. A 4 % degradation was observed including area increment of previously present impurities and formation of new impurities. The Agilent Poroshell 120 EC-C8 column offered excellent chromatographic separations of the majority of impurity peaks with few coelutions.

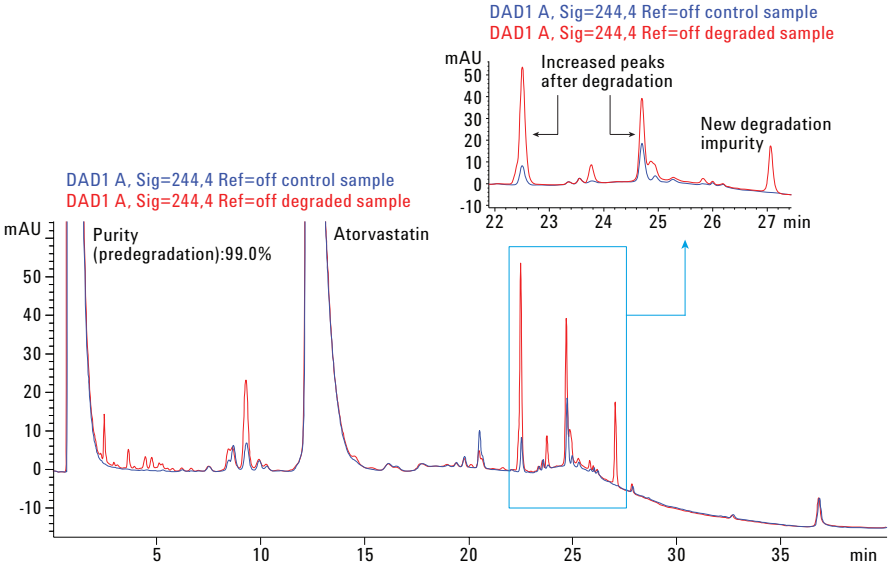


Figure 2. Monitoring the extent of degradation using an HPLC-UV method. UV trace at 244 nm of nondegraded (blue) and degraded (red) samples are overlaid. A portion of the LC chromatogram is zoomed in to demonstrate the formation of new and increased impurity peaks.

Accurate mass screening of impurities

The HPLC/Q-TOF analysis in both positive and negative ion modes confirmed the presence of degradants. Figure 3 shows an overlay of total ion chromatograms (TICs) of stable and degraded samples.

Impurity identification

The Batch Recursive Feature Extraction algorithm of Agilent MassHunter Profinder processed degraded and nondegraded sample data files. Results were exported to Mass Profiler Professional (MPP) for fold change analysis. A screenshot from the MassHunter Profinder showing EICs and spectra of impurity with mass 465.1589 is given in Figure 4. The Profinder results show that impurity with mass 465.1589 is formed during degradation, since this peak was absent in the blank and nondegraded samples. Multiple runs confirmed these observations.

The four-way Venn diagram in Figure 5 graphically depicts impurities between nondegraded and degraded samples (both positive and negative mode). Fold change analysis of the results from degraded and nondegraded samples showed that approximately 15 impurities had ≥ 2-fold changes. Figure 6 shows a Venn diagram between degradants found in positive and negative mode. Seven impurities increased in the positive mode, and eight impurities increased in the negative mode. Of the 15 impurities, two up-regulated impurities were common.

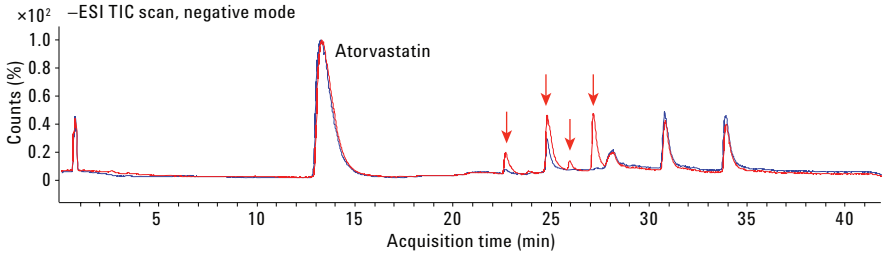


Figure 3. Overlay of TIC for nondegraded sample and degraded sample. A few significant peaks observed in TIC of degraded samples are marked.

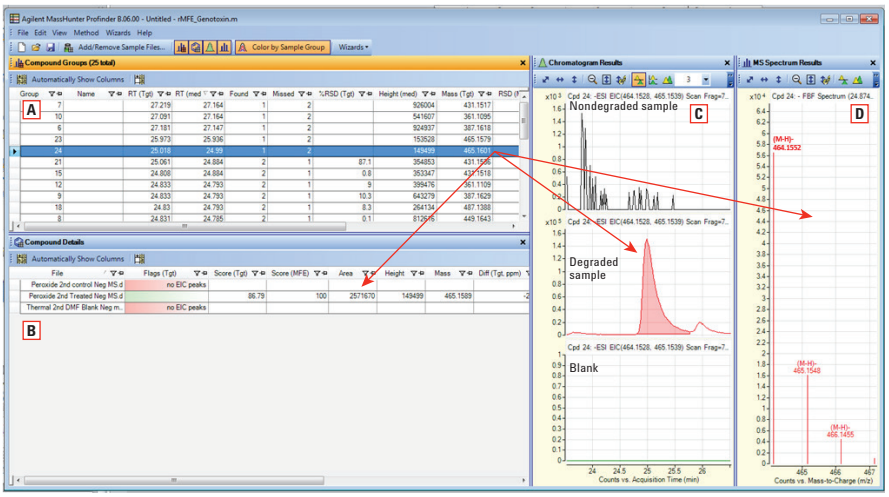


Figure 4. Screenshot of Agilent MassHunter Profinder result, selected for an unknown impurity with molecular weight M = 465.1589. Panel A is for Compound Group Table where compound features are aligned by neutral mass and retention time. Panel B is Compound Detail Table, where details like individual scores, flags, abundances. for each individual feature are displayed. Panel C is for displaying Extracted Ion Chromatograms (EICs) for the selected *m/z* from blank, nondegraded and degraded traces. Panel D is for displaying corresponding mass spectrum of selected ion.

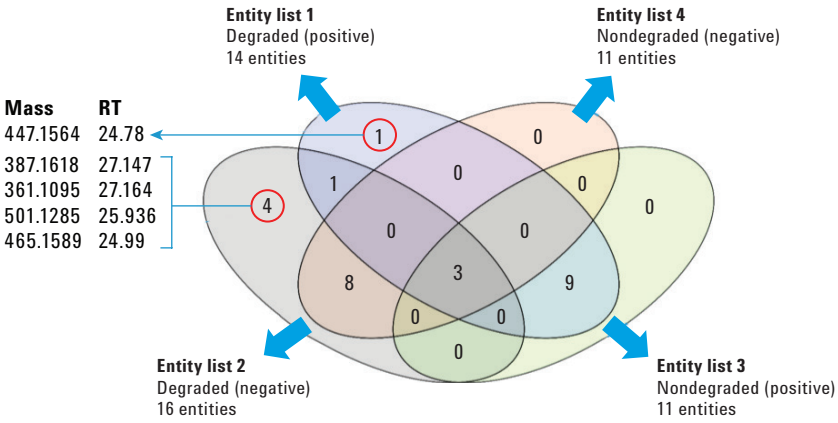
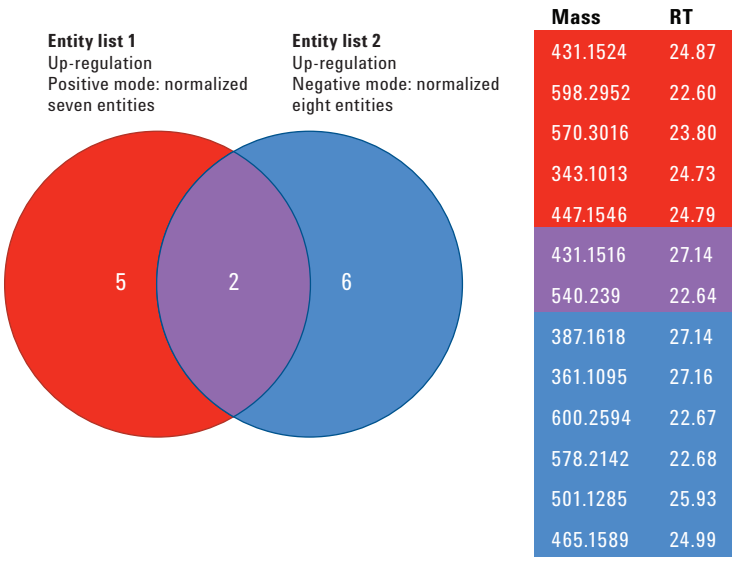


Figure 5. Four-way Venn diagram showing impurity distribution between stable and degraded samples. The unique impurities observed from degraded sample in LC/MS positive and negative mode are marked in red circles.

Accurate mass data of those 15 impurities were searched against the accurate mass personal compound database (PCD), which resulted in the identification of five known impurities. This includes oxidative products of atorvastatin suggested by Mass-MetaSite Software.

Q-TOF MS/MS data was processed using the MFE algorithm to extract MS and MS/MS information of remaining unidentified impurities. This information was imported into the MSC software to get putative structures for each unknown impurity. MSC calculates all possible subformulas for each fragment ion with mass deviation in ppm. Figure 7 contains a screenshot of the MSC results for an unknown impurity with mass 465.1578 ($C_{26}H_{24}FNO_6$). The results show a potential structure containing epoxide and hydroperoxide functional groups. All search results were obtained within 10 seconds.

After identifying the structures of all 15 impurities, three of them were found to contain genotoxic epoxide and hydroperoxide functional groups. The proposed alerting structures are given in Figure 8 (Impurities I, II, and III). These structures were confirmed by using references[1,2,9]. Impurities I and II are listed in the United States Pharmacopeia, while Muhammad Ashfaq *et al.* suggested Impurity III as a possible impurity formed upon oxidation[9]. (From LC/UV data, the percentage area for Impurity III was ≤ 0.1 %).



Mass	RT
431.1524	24.87
598.2952	22.60
570.3016	23.80
343.1013	24.73
447.1546	24.79
431.1516	27.14
540.239	22.64
387.1618	27.14
361.1095	27.16
600.2594	22.67
578.2142	22.68
501.1285	25.93
465.1589	24.99

Figure 6. A Venn diagram between impurities found in positive and negative mode with a fold change > 2. From the listed retention times (RTs), few impurities were found to be coeluting.

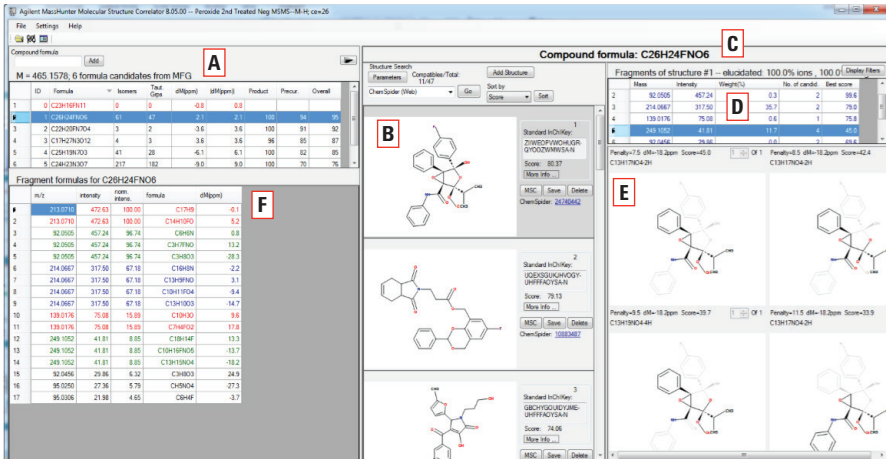


Figure 7. Screenshot of MSC result for the unknown impurity with mass 465.1578.
A) MFG score based on accurate mass of each MS/MS fragment ion and its neutral loss from precursor
B) Proposed alerting structure is in the 1st position
C) 100% of the total fragment ion intensity could be explained by the MSC, resulting in an overall score of 80.37.
D) Multiple candidate isomeric structures are possible, for example, two for 214.0667.
E) Showing individual substructures with penalties

Structure comparison with MS/MS pattern matching

The MS/MS fragmentation pattern of the newly identified Impurity III (mass 465) was compared with the known Impurity II to verify the structure (Figure 9). The fragmentation pattern of Impurity III with *m/z* 464.1505 (negative mode, $(M-H)^-$) has a similar pattern to Impurity II (*m/z* 448.1656), which supports the MSC results. The difference between Impurity II and III is just an oxygen atom in the hydroperoxide functional group.

Isolation of genotoxic impurities

The newly formed alerting Impurities I and III were collected in a mass-triggered fraction collection, where an active splitter diverted the flow to the mass spectrometer. LC/SQ analyzed the purities of the collected fractions (Figure 10) and they were found to be very pure. This process can be scaled up or pooled from multiple injections for further structure confirmation using techniques such as NMR.

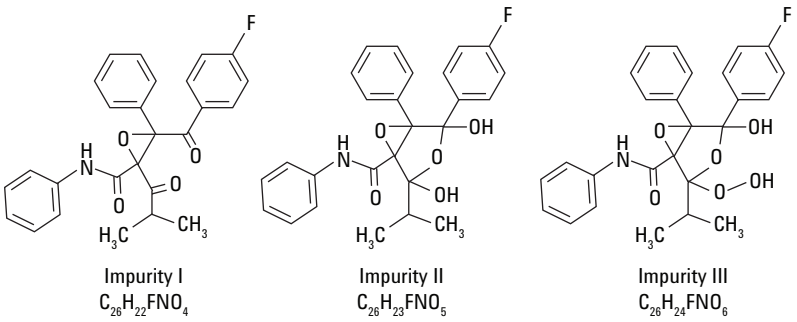


Figure 8. Impurities (I, II, and III) having alerting structures (epoxide and hydro peroxide functional groups) found in atorvastatin oxidation degradation sample.

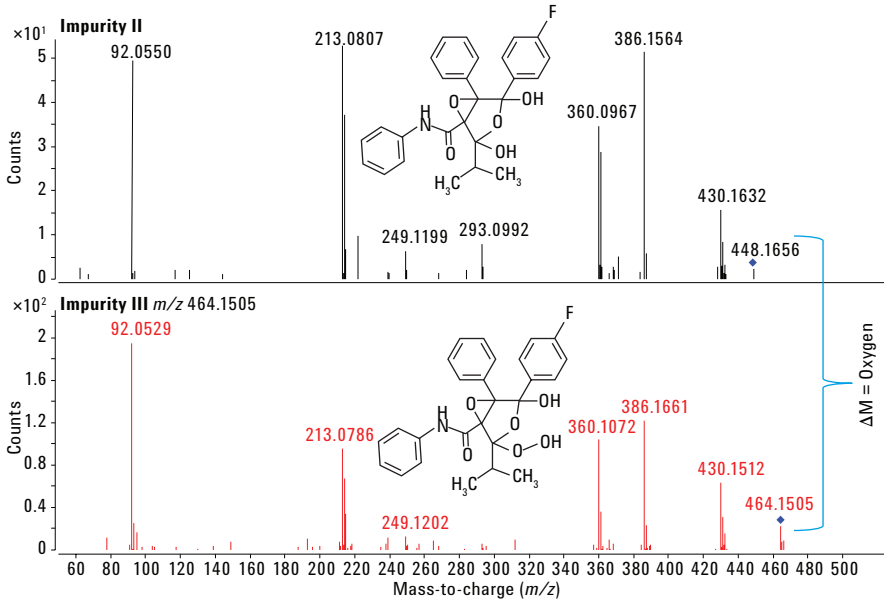


Figure 9. Fragmentation pattern comparison of newly found alerting Impurity III having M: 465.4702 (bottom trace) with known alerting Impurity II, M: 449.4708 (top trace) in negative mode.

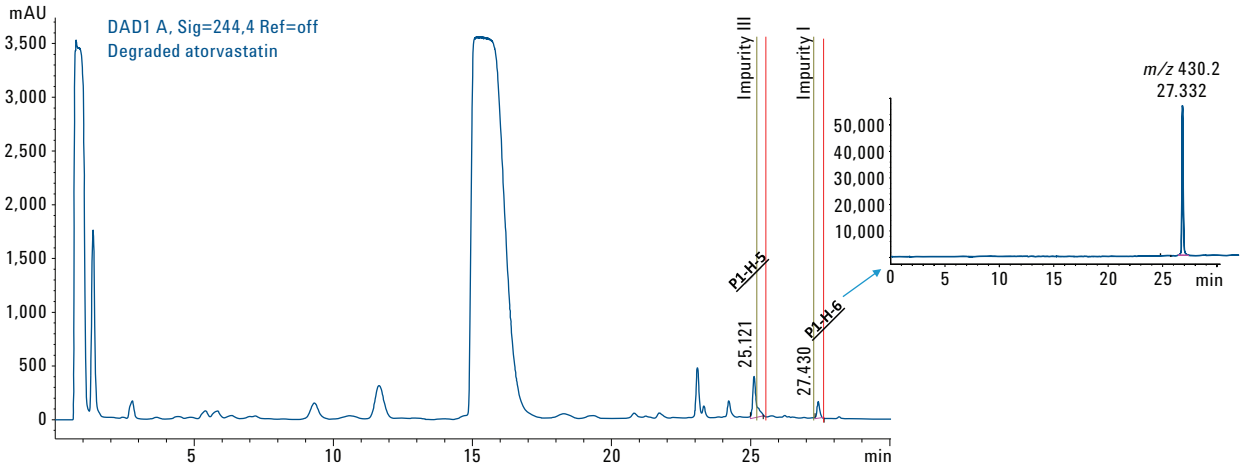


Figure 10. Fraction collection of alerting impurities (Impurities I and III) using mass based approach. Purity of the collected fractions were verified using LC/SQ analysis and result of Impurity I is shown in inset as an example.

Conclusion

This Application Note demonstrates structural identification of possible potential genotoxic impurities originating from forced degradation of atorvastatin drug substance. The workflow includes screening the degraded sample by LC/UV and accurate mass Q-TOF LC/MS. LC/UV analysis estimates the extent of degradation, and Q-TOF LC/MS analysis collects accurate mass information for all the impurities. Accurate mass data is processed using various Agilent MassHunter software solutions to identify known impurities, and propose possible structures for unknown impurities. The formation of a new genotoxic alerting impurity (epoxide impurity) is identified from the MSC proposed structures. MS/MS fragment-pattern match with a known structurally similar impurity can be performed to support the findings. Agilent LC/MS analytical fraction collection system is used to collect the potential genotoxic impurities and verify the purity of collected fractions. This study also proves that thermally labile genotoxic impurities such as epoxides can be easily monitored using an Agilent Jet Stream source.

Acknowledgment

We thank Dr. Roy Helmy, Merck & Co., Inc. for his immense technical support in designing and executing the project.

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Published in the USA, May 1, 2014
5991-4404EN

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