Pharmaceutical Impurity Identification and Profiling Using Agilent Q-TOF LC/MS Combined with Advanced MassHunter Data Processing Software

Application Note

Authors

Siji Joseph
Agilent Technologies
LSCI Bangalore, India

Yuqin Dai
Agilent Technologies, Inc.
Santa Clara, CA, USA

Abstract

This application note describes an efficient software-assisted workflow for impurity identification and profiling of active pharmaceutical ingredients (API) using a high resolution accurate mass (HRAM) quadrupole time-of-flight (Q-TOF) LC/MS system. The workflow involves two steps: on-line LC-UV detection, followed by MS and auto MS/MS analysis; and identification and structure elucidation of impurities using advanced MassHunter Qualitative Analysis data processing algorithms such as Molecular Feature Extraction (MFE) and Molecular Formula Generation (MFG), and Molecular Structure Correlator (MSC) software. The Agilent 6540 Ultra High Definition Accurate Mass Q-TOF LC/MS System provides sensitive MS and MS/MS analysis of trace-level impurities in drug substances with sub-ppm mass accuracy. The effective use of this workflow for impurity profiling is demonstrated by the rapid identification and structural elucidation of atenolol and eight European Pharmacopoeia (EP) specified impurities, including genotoxic impurity D and two isomers of impurity F at the levels of 0.02 to 0.07 % relative to the atenolol UV detection area.

Agilent Technologies

Introduction

Impurity identification and profiling is critical to the assurance of patient safety and drug efficacy in a drug development and API manufacturing unit. Regulatory authorities have established clear and rigorous guidelines which dictate the identification of impurities at lower levels depending upon dosage. As per United States Food and Drug Administration (FDA) recommendations, any impurities having an area percentage >0.05 of API should be reported.[1] Currently, various analytical techniques are available for the identification and quantification of drug impurities. However, identification of trace levels of impurities is still challenging, as conventional analytical approaches often involve multiple instrument platforms and sample workup steps such as purifying/collecting a specific impurity by preparative LC followed by lyophilization, and performing NMR analysis, which can be time-consuming and laborious.

The objective of this application note is to develop a streamlined workflow to effectively identify impurities using an Agilent LC UV/Q-TOF MS system in combination with advanced data processing software. An example of identification and structure elucidation of eight atenolol impurities at levels of 0.02 to 0.07 % is demonstrated.

Experimental

Figure 1 shows a novel workflow for impurity identification and profiling. This workflow has been streamlined to provide high-confidence, accurate identification and faster structure elucidation compared to conventional impurity profiling, which requires multiple platforms and spreads analysis over multiple days.

Instrument

The LC/MS system consisted of an Agilent 6540 UHD Q-TOF with a Jet Stream source, and an Agilent 1200 Series Binary LC System, which consists of following modules: Agilent 1200 Series degasser (p/n G1379B), Agilent 1200 Series Binary Pump (p/n G1312B), Agilent 1200 Series High-Performance Autosampler (p/n G1367D), Agilent 1200 Series Thermostatted Column Compartment (p/n G1316B), and an Agilent 1290 Infinity Diode Array Detector (G4212A) with Max-Light flow cell, (4.0 µL volume, 60-mm path length) (G4212 A). The software included an Agilent MassHunter Workstation (version B.04.00) for data acquisition, MassHunter Qualitative Analysis software (B.04.00) for data analysis, and MassHunter MSC software (version B.05.00) to facilitate the elucidation of impurity structures.

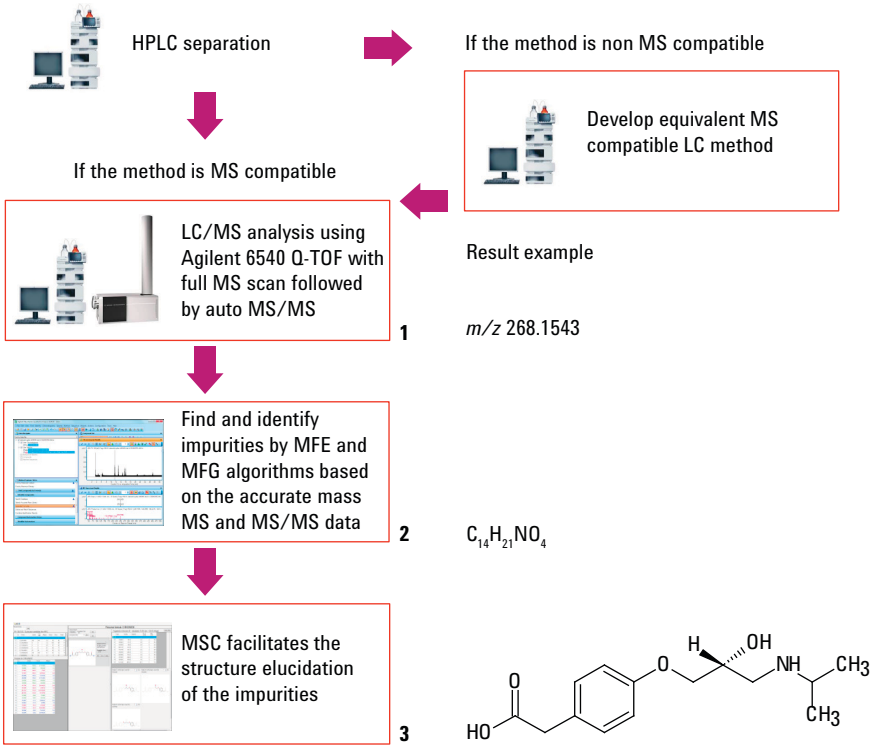

Figure 1. Software assisted workflow for impurity identification and profiling of pharmaceuticals using an Agilent 6540 UHD Q-TOF LC/MS, MassHunter Qualitative Analysis, and MSC software.

Reagents and materials

LC/MS grade methanol and formic acid were purchased from Fluka (Germany). Highly purified water from a Milli Q system (Millipore Elix 10 model, USA) was used for mobile phase preparation. All other reagents used to execute the pharmacopeia HPLC method for atenolol were purchased from Aldrich (India). Standards of atenolol API and eight impurities (A to H) were purchased from LGC Promochem (Germany). The structure of atenolol and eight impurities are shown in Figure 2 and molecular details are listed in Table 1.

LC/MS conditions

The LC/MS conditions were optimized and are summarized in Tables 2 and 3.

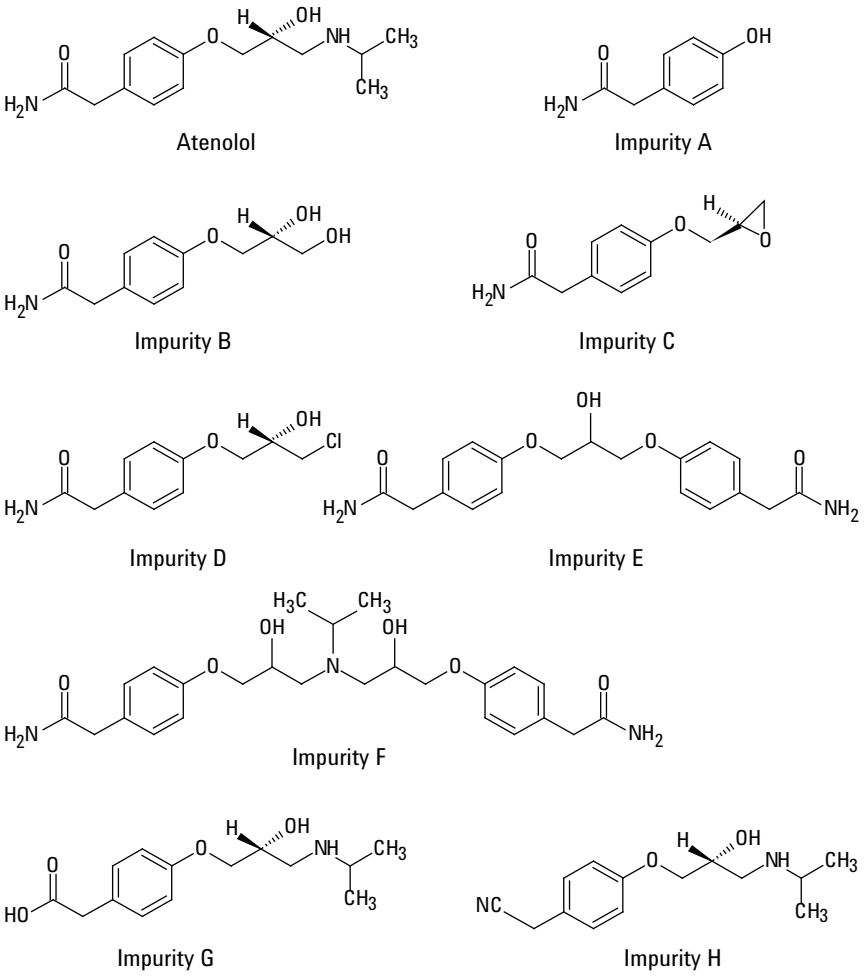

Figure 2. Molecular structures of atenolol and eight impurities as per EP (6.0).

Table 1. Molecular details of atenolol and eight impurities.

Name	Molecular formula	Molecular weight	$(M+H)^+$
Atenolol	$C_{14}H_{22}N_2O_3$	266.3361	267.1703
Impurity A	$C_8H_9NO_2$	151.1626	152.0706
Impurity B	$C_{11}H_{15}NO_4$	225.2411	226.1074
Impurity C	$C_{11}H_{13}NO_3$	207.2258	208.0968
Impurity D	$C_{11}H_{14}ClNO_3$	243.6868	244.0735
Impurity E	$C_{19}H_{22}N_2O_5$	358.3884	359.1601
Impurity F	$C_{25}H_{35}N_3O_6$	473.5619	474.2599
Impurity G	$C_{14}H_{21}NO_4$	267.3208	268.1543
Impurity H	$C_{14}H_{20}N_2O_2$	248.3208	249.1598

Note: The $(M+H)^+$ values were calculated using MassHunter Mass Calculator

Table 2. LC conditions.

LC (MS compatible) conditions			LC (EP) conditions
Column	Agilent Poroshell 120 EC-C18, 3.0 × 150 mm, 2.7 µm (p/n 693975-302)		Agilent ZORBAX ODS 4.6 × 150 mm, 5 µm (p/n 883952-702)
Flow rate	0.4 mL/min		1 mL/min
Mobile phase A	0.1% formic acid in water		1 g of sodium octane sulfonate + 0.4 g of tetrabutylammonium hydrogen sulphate in 2:18:80 of tetrahydrofuran:methanol:buffer. Buffer 3.4 g /L solution of KH_2PO_4, adjust the pH to 3.0 with H_3PO_4
Mobile phase B	0.1 % formic acid in methanol		Not applicable
Detection	226 nm		226 nm
Injection volume	2 µL		10 µL
Needle wash	Activated for 5 seconds using methanol		Activated for 6 seconds using methanol
Pump mode	Gradient		Isocratic
	Time	% B	100 % A from 0 to 30 minutes
	0	11	
	25	30	
	26	30	
	36	11	
Post run	5 minutes		Not applicable
Column temperature	43 °C		Not maintained

Table 3. Agilent 6540 UHD Q-TOF parameters.

Q-TOF MS and auto MS/MS conditions	
Ion source	AJS ESI
Acquisition mode	2 GHz, Ext dynamic range
Ion polarity	$^{+}$ve mode
Data storage	Both centroid and profile
Drying gas temperature	350 °C
Drying gas	10 L/min
Nebulizer	45 psig
Sheath gas temperature	400 °C
Sheath gas flow	12 L/min
VCap	4,000 V
Nozzle voltage	500 V
Fragmentor	150 V
Skimmer	75 V
OCT 1RF Vpp	750 V
Acquisition	MS followed by auto MS/MS
MS acquisition rate	5 spectra/s
MS/MS acquisition rate	4 spectra/s
Isolation width	Medium (~4 *m/z*)
Collision energy, use formula	Slope 4, offset 10

Sample preparation and analysis

Atenolol was spiked with the eight EP specified impurities. The concentration of atenolol in the spiked sample is approximately 2,000 ppm and the percentages of impurities in atenolol range from 0.02 to 0.07 %. The spiked sample was initially analyzed using the EP method[2] and the percentage area of each impurity was measured. Since the pharmacopoeia HPLC method involves nonvolatile buffers which are not compatible with mass spectrometry (MS) analysis, an MS compatible LC method was developed using water/methanol containing 0.1 % formic acid as mobile phase. The sample was analyzed by on-line UV detection followed by full MS and auto MS/MS analysis with the 6540 UHD Q-TOF LC/MS System.

Results and Discussion

LC-UV and LC-UV/6540 UHD Q-TOF MS analysis

The chromatographic separation of a spiked atenolol sample, using the EP method, is shown in Figure 3. Figure 4 shows separation of the same sample using the MS-compatible LC method where all eight impurities are well separated. Although the elution order of each impurity was found to be different in the LC/MS method from the EP method, percentage areas of all impurities from both methods were comparable. Table 4 summarizes the observed area percentages of atenolol and impurities from the LC/MS method.

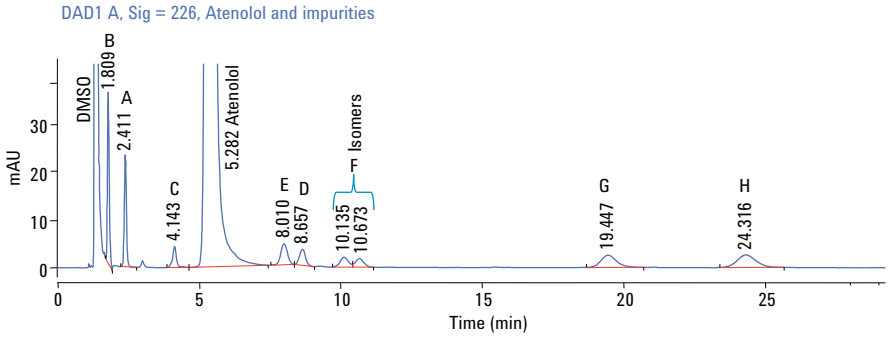

Figure 3. Chromatographic separation of atenolol and eight impurities using the EP method.

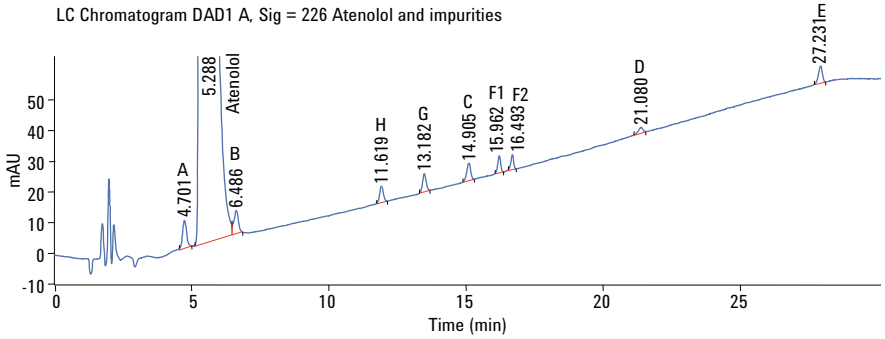

Figure 4. Chromatographic separation of atenolol and eight impurities using an in-house developed MS compatible method.

Table 4. Percentage areas based on the UV signals.

Peak no.	Time	Area%	Name
1	4.70	0.07	A
2	5.28	99.61	Atenolol API
3	6.48	0.07	B
4	11.61	0.04	H
5	13.18	0.04	G
6	14.90	0.04	C
7	15.96	0.03	F1
8	16.49	0.03	F2
9	21.08	0.02	D
10	27.23	0.05	E

Figure 5 shows the Total Ion Chromatogram (TIC). The LC eluants from 0 to 3.5 minutes and from 5.5 minutes to 6.0 minutes were diverted to the waste.

Data analysis using Agilent MassHunter Qualitative Analysis software

Data analysis was performed using the MFE and MFG algorithms of the MassHunter Qualitative Analysis software. The MFE algorithm was able to automatically locate all sample components in an untargeted fashion and to extract all relevant spectral and chromatographic information for the trace impurity entities. The MFG algorithm takes full advantage of the mass accuracy of the data. After user input (allowed elements, even/odd electrons, and charge carrier) based on the knowledge of sample composition, the MFG uses information at the MS level (masses of main isotope, isotope abundances, and isotope spacing) and the MS/MS level (masses of MS/MS fragment ions and neutral losses if available), for the calculations to generate empirical formulas. Using all this accurate mass information results in a smaller and more relevant list of candidate molecular formulas for each entity and ranks them according to the relative probabilities (maximum score is 100 %).

Figure 6 shows the measured isotope pattern versus a theoretical one of impurity B (the latter shown in rectangles), and MFG scores and mass accuracy for each isotope, demonstrating the high isotopic fidelity with match scores greater than 99 % for masses, isotope abundance, and isotope spacing. These results provide reliable formula generation and impurity identification with high confidence.

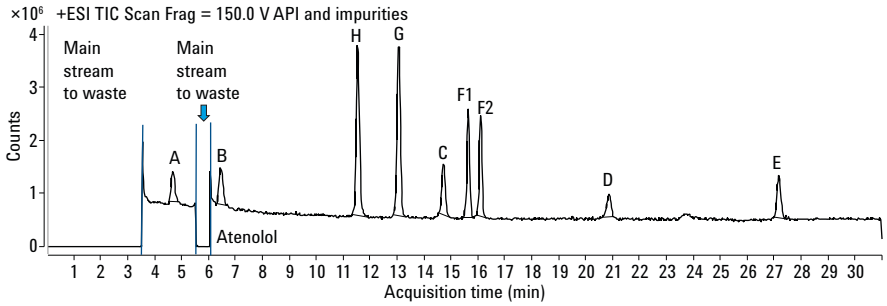

Figure 5. Total ion Chromatogram (TIC) using on-line LC/UV detection and 6540 UHD Q-TOF MS analysis.

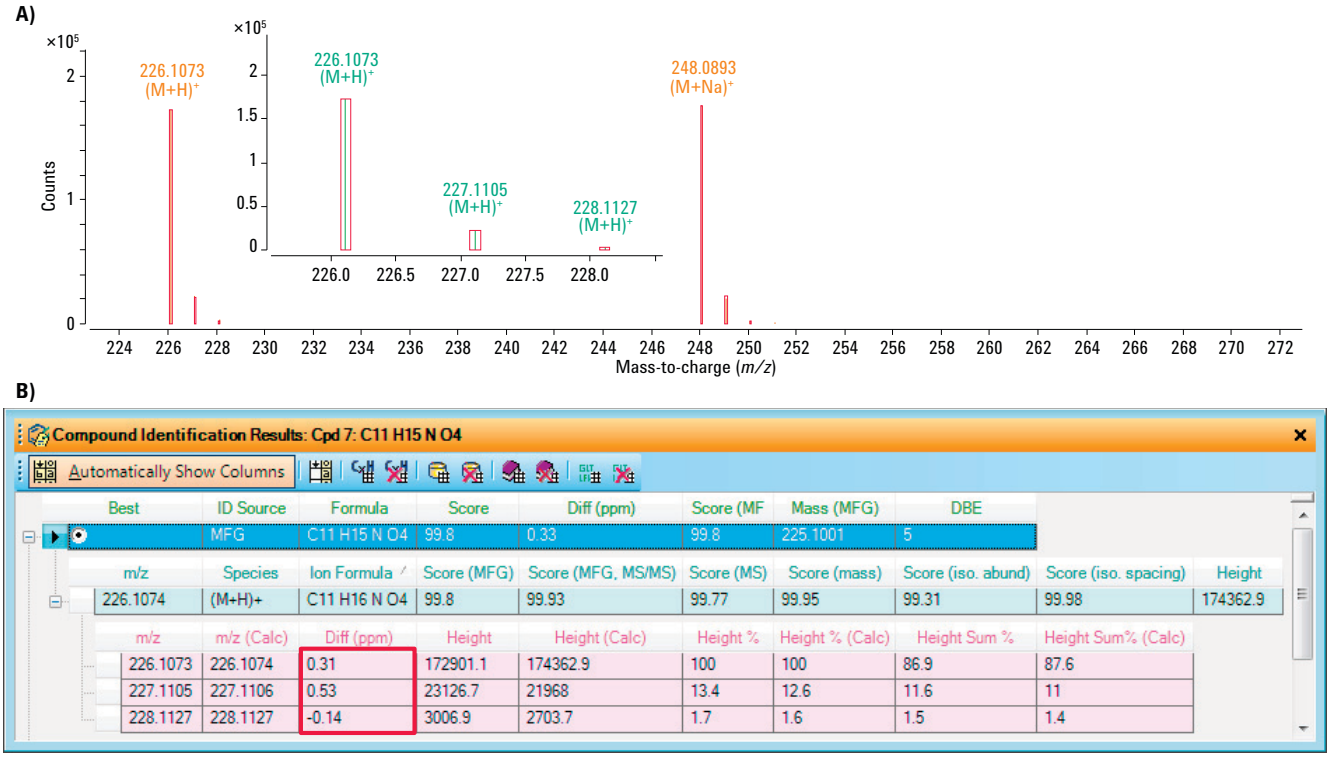

Figure 6. (A) shows the MS spectrum of impurity B, and (B) shows the MFE/MFG results for impurity B.

Table 5 summarizes the results from MFE and MFG algorithms for all eight impurities and atenolol. The compound numbers are labeled based on the retention time (RT) in the order the impurities are eluted. Two hits were observed for atenolol, representing the leftover beginning and end of the atenolol main peak, which was diverted to the waste. Isomers of Impurity F were chromatographically separated and are listed as impurity F1 and F2. As illustrated in Table 5, the observed $(M+H)^+$ values correlate very well with the theoretical $(M+H)^+$, with mass errors of less than 1 ppm for all impurities identified.

Agilent MassHunter Molecular Structure Correlator (MSC) software

MSC correlates the accurate mass of MS/MS fragment ions of a compound of interest with one or more proposed molecular structures for that compound. MSC accomplishes this by correlating each observed fragment ion to the proposed structure using a systematic bond-breaking approach. An overall correlation score is calculated from individual scores for each fragment ion signal. For each fragment ion, one or multiple substructure candidates may be suggested (Figure 8) and a penalty is assigned based on the number and type of bonds needed to be cleaved to generate that substructure, as well as the number of hydrogen atoms that need to be added or subtracted to explain the observed fragment ion mass. For example, breaking two bonds, or a double bond or even an aromatic ring carries a higher penalty than just breaking one single bond. Two other factors impacting the overall correlation score are the mass accuracy of the observed fragment ions and the overall percentage of fragment ion intensity that can be explained with substructures.[3] MSC software can be used to confirm a user-proposed structure for a compound and also aid in the identification of true unknowns.

Table 5. Molecular formulas obtained from MFE/ MFG results.

Cpd	Formula from EP	Formula from MFG	Score (MFG)	Theoretical $(M+H)^+$	Experimental $(M+H)^+$	Mass error (ppm)
1 Imp A	$C_8H_9NO_2$	$C_8H_9NO_2$	99.78	152.0706	152.0706	0.00
2 Atenolol	$C_{14}H_{22}N_2O_3$	$C_{14}H_{22}N_2O_3$	98.68	267.1703	267.1704	0.37
3 Atenolol	$C_{14}H_{22}N_2O_3$	$C_{14}H_{22}N_2O_3$	99.48	267.1703	267.1705	0.75
4 Imp B	$C_{11}H_{15}NO_4$	$C_{11}H_{15}NO_4$	99.42	226.1074	226.1073	0.44
5 Imp H	$C_{14}H_{20}N_2O_2$	$C_{14}H_{20}N_2O_2$	99.79	249.1598	249.1599	0.40
6 Imp G	$C_{14}H_{21}NO_4$	$C_{14}H_{21}NO_4$	99.39	268.1543	268.1545	0.75
7 Imp C	$C_{11}H_{13}NO_3$	$C_{11}H_{13}NO_3$	99.01	208.0968	208.0966	0.96
8 Imp F1	$C_{25}H_{35}N_3O_6$	$C_{25}H_{35}N_3O_6$	99.17	474.2599	474.2601	0.42
9 Imp F2	$C_{25}H_{35}N_3O_6$	$C_{25}H_{35}N_3O_6$	98.35	474.2599	474.2599	0.00
10 Imp D	$C_{11}H_{14}ClNO_3$	$C_{11}H_{14}ClNO_3$	98.23	244.0735	244.0736	0.41
11 Imp E	$C_{19}H_{22}N_2O_5$	$C_{19}H_{22}N_2O_5$	98.36	359.1601	359.1604	0.84

The utility of MSC for structure elucidation of impurity E and F are demonstrated in Figures 7 and 8.

Figure 7 shows how the MSC software can aid in identification of an unknown impurity. The accurate masses of the precursor ion and fragment ions of impurity F were used to calculate the most probably molecular formula, which then was searched against the ChemSpider database to retrieve all possible structures. Multiple candidate structures were returned with their calculated correlation scores. The primary MSC proposed structure with the highest correlation score of 99.14 % matches exactly to that of impurity F. The overall MFG score for the selected precursor ion, the rank of the MSC proposed structure and the structure correlation score for impurity F are highlighted in red circles.

Figure 8 illustrates how the MSC software can be used to confirm a user-proposed structure. The MS/MS spectral data of impurity E was uploaded to the MSC software, and the structure of Impurity E was imported as a ".mol" file. As shown in the Figure 9, the proposed structure correlates very well with the MS/MS spectrum of Impurity E with a correlation score of 97.35% (highlighted in red circle).

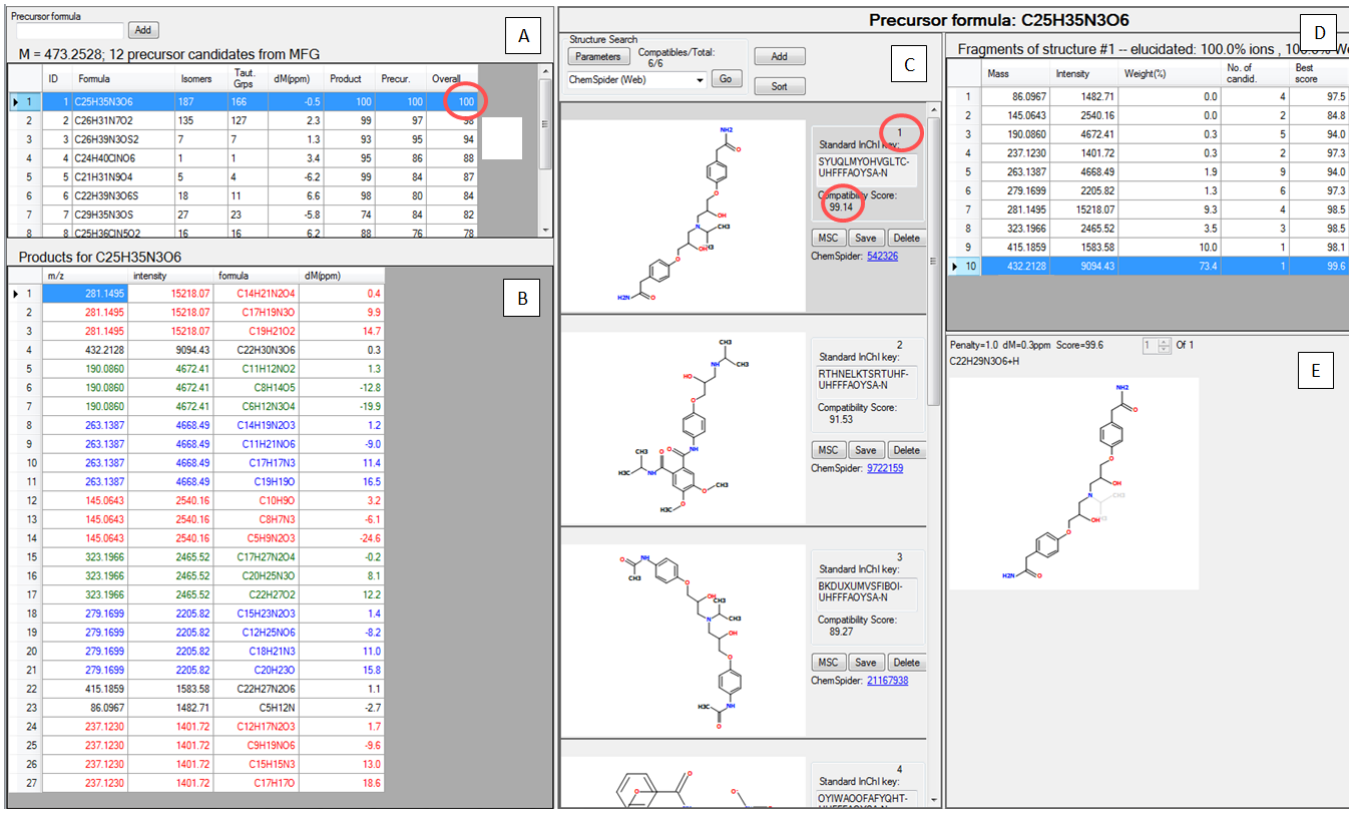

Figure 7. Screen shot of MSC results for Identification of an unknown impurity (Impurity F). List of possible molecular formulas for the precursor ion for Impurity F (A), MFG results of product ions for a selected precursor candidate in panel A (B), Candidate structures for the parent compound (C), Fragment ions for the candidate structure selected in panel C (D), and Substructure assignments for a selected fragment ion in panel D (E).

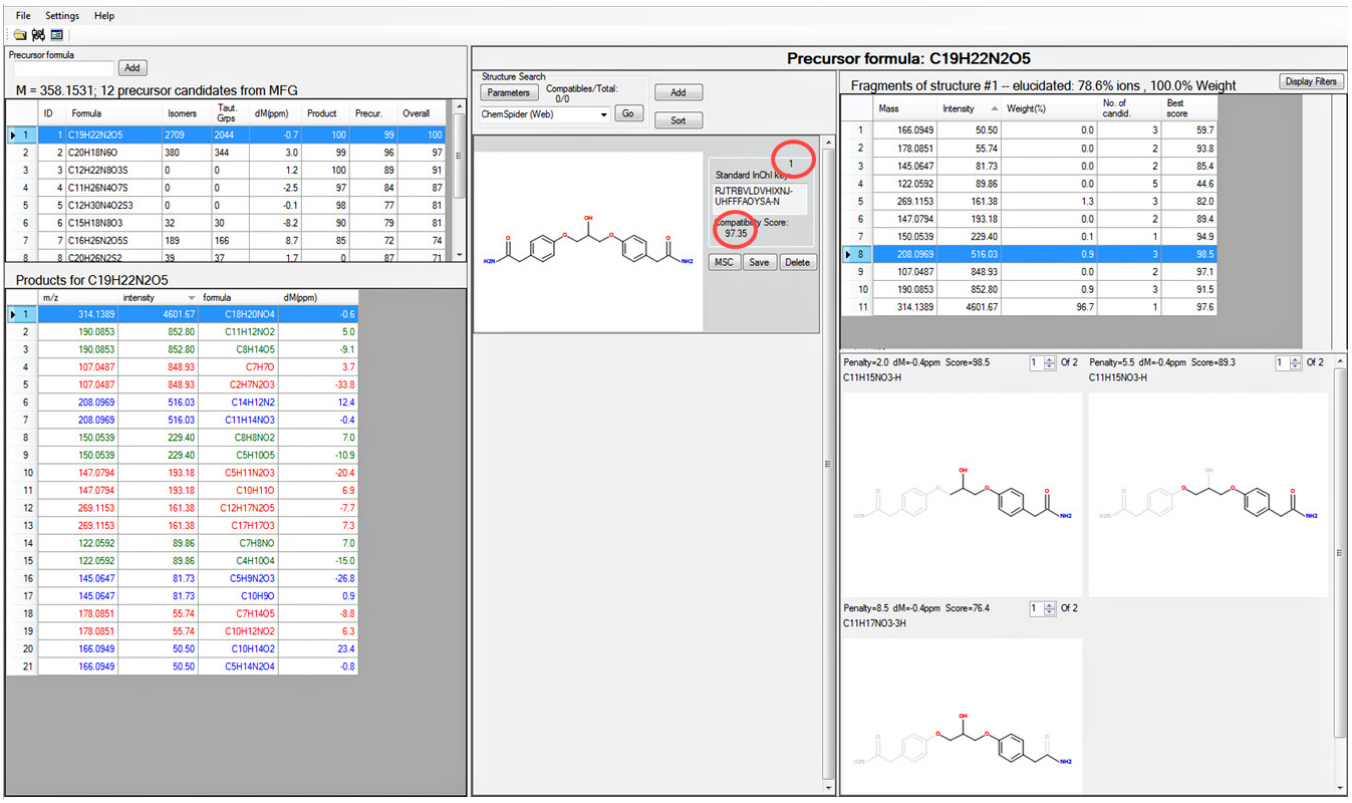

Figure 8. Screen shot of MSC results for confirmation of a proposed structure (Impurity E).

Using these approaches, the structures of all the remaining entities/impurities were elucidated. The molecular structures of the atenolol impurities obtained using MSC software correlate with the validated structures in Pharmacopeia (Figure 2). Table 6 summarizes the results from the MSC.

Table 6. MSC results for atenolol API and its impurities.

No.	Entity	Precursor dM (ppm)	Compound correlation/ compatibility score (%)	% Weight*
1	Atenolol API	-1.1	94.97	100
2	Impurity A	-0.6	96.0	99.4
3	Impurity B	0.4	94.93	100
4	Impurity C	0.6	92.18	99.8
5	Impurity D	-2.1	90.83	98.7
6	Impurity E	-0.7	97.35	100
7	Impurity F	-0.5	99.14	100
8	Impurity G	-0.6	96.20	100
9	Impurity H	-0.6	97.73	100

*% of total ion intensity explained, weighted by the mass of the fragment ion. (Explanation of higher mass fragment ions represent higher evidence.)

Figure 9 shows the MS/MS spectra of atenolol and impurity G. By comparing the *m/z* values of the precursor and fragment ions of both atenolol and impurity G, as well as the proposed substructures by MSC software for each compound, the degradation site of the impurity G can be easily determined.

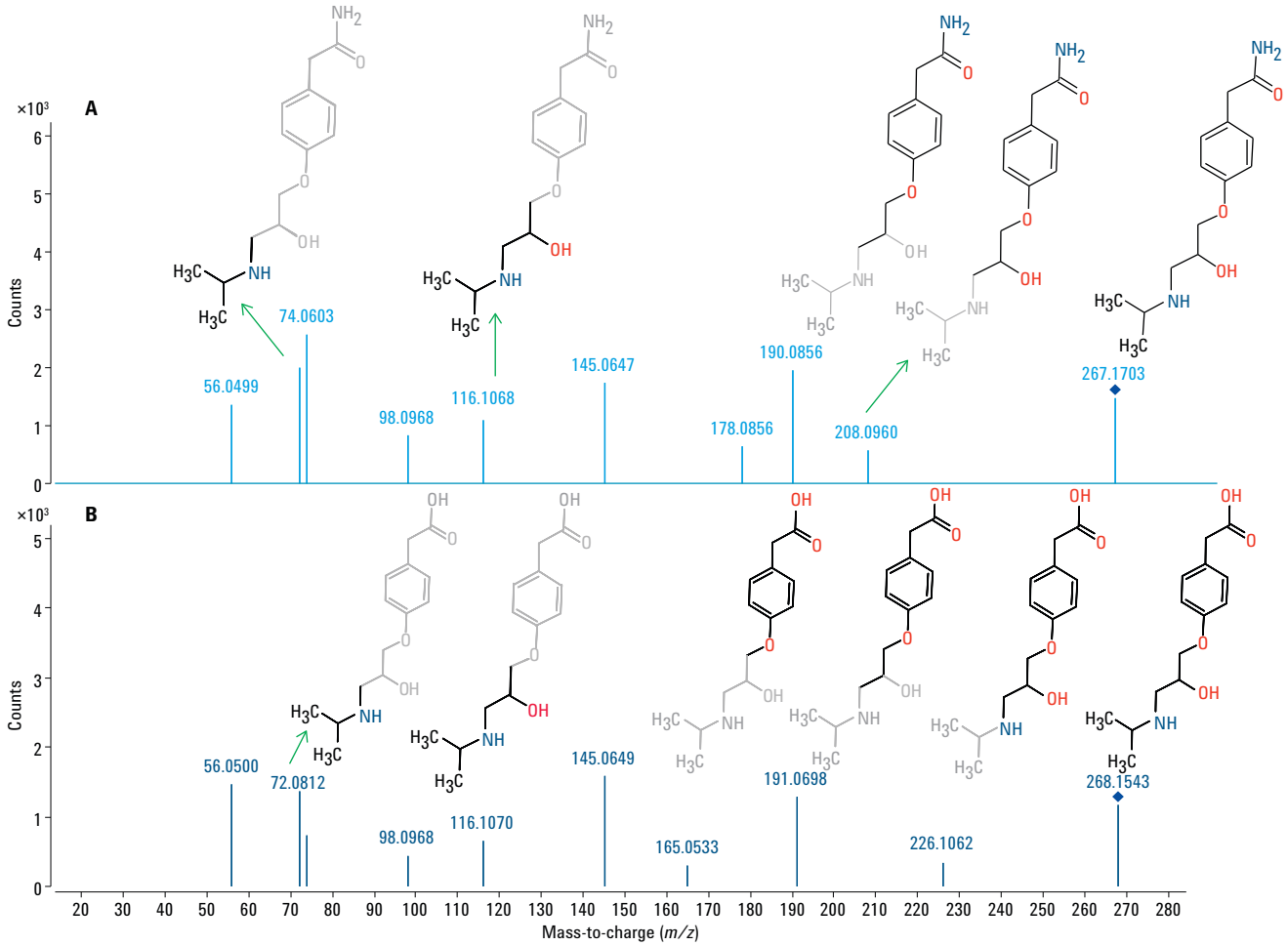

Figure 9. Structure elucidation of impurity G assisted by MSC software. MS/MS spectra of atenolol (A) and Impurity G (B).

Conclusion

An efficient workflow was developed for identification and profiling of trace levels of pharmaceutical impurities using Q-TOF technology combined with advanced data processing software. The wide in-spectrum dynamic range of the Agilent 6540 UHD Q-TOF allowed identification of all eight trace level impurities from an atenolol API sample. A high MFG score of greater than 98.0 and low mass error of less than 1 ppm were achieved, leading to highly confident impurity identification. MFE, MFG, and MSC proved to be essential tools for the fast and efficient identification and structure determination of impurities. Furthermore, MSC can be efficiently used to narrow down the number of structural possibilities of unknown impurities, providing insight into the substructures that might exist in an unknown molecule or to suggest the class of molecules.

References

1. Guidance for Industry Q3A Impurities in New Drug Substances, June 2008, Revision 2.

2. European pharmacopoeia 6.0, (2008), Volume 2, page 1228-1229(4).

3. Agilent MassHunter Molecular Structure Correlator (MSC) Software Quick Start Guide, Agilent Publication G3335-90126.

www.agilent.com/chem/QTOF

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