Identification and Structural Elucidation of Amlodipine Impurities Using High Resolution LC/MS and LC-MS/MS

Application Note

Pharmaceutical

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Abstract

Characterization of impurities in drug products is an essential requirement in the pharmaceutical development process. The identification of impurities and their structural elucidation is greatly facilitated using high resolution accurate mass LC/MS together with comprehensive software tools for data processing and structural characterization. An Agilent 1290 Infinity LC system coupled to an accurate mass Agilent 6540 Q-TOF system was used for the structural elucidation of in-process amlodipine impurities. Available amlodipine impurity standards were used to create an accurate mass MS database together with MS/MS and pseudo MS^3 spectral libraries using the Agilent Personal Compound and Database Library (PCDL) software. Agilent Molecular Structure Correlator (MSC) software was also used to identify substructures and aid confirmation of impurities. The accurate mass MS, MS/MS, and pseudo MS^3 spectra of unknown in-process impurities were then searched against the known libraries to identify common substructures. Elemental compositions and associated structures for the impurities could then be proposed. Again, with the help of MSC and ACD software (ACD labs), the fragmentation pathway of the proposed structure was established.

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Introduction

Pharmaceutical regulatory authorities require identification and structural elucidation of impurities that exceed toxicity or detection thresholds. Identification is facilitated by mass or formula database searches. Structure elucidation is facilitated by nominal mass or high-resolution LC/MS instruments capable of MS/MS or MS^3 acquisitions. While low-resolution nominal mass quadruple instruments can be used for structural analysis, the use of high-resolution TOF technology greatly increases the confidence with which compound identification can be made. High-resolution accurate mass quadrupole time-of-flight mass spectrometers acquire accurate mass MS, MS/MS, and pseudo MS^3 spectra. In pseudo MS^3 acquisition mode, the source voltage can be varied to cause precursor fragmentation prior to MS/MS analysis. The accurate mass of each fragment can then be used to calculate elemental composition and fragmentation pathways to aid structural elucidation.

In-process impurities often are structurally related to process ingredients or intermediates. Therefore, a direct comparison of accurate mass MS, MS/MS, and pseudo MS^3 of unknowns with known standards facilitates structural elucidation. Additionally, the use of intelligent software tools such as the Agilent Molecular Structure Correlator (MSC) software, helps to determine substructures and elucidation of fragmentation pathways. MSC software proposes possible fragmentation mechanisms by correlating accurate mass and elemental formulae of MS/MS fragments with possible *in silico* fragment ions from proposed structures.

In this work, the structure of unknown, in-process impurities of amlodipine, a calcium channel blocker used for controlling blood pressure, was elucidated using an Agilent 1290 Infinity LC system coupled to an Agilent Accurate Mass 6540 Q-TOF System. Figure 1 shows the methodology used in this work.

Here, a custom spectral library of known impurity standards was created using the Agilent Personal Compound and Database Library (PCDL) software. Using the fragment search feature of PCDL and fragmentation site comparison with known impurities, the structure of unknown impurities were proposed and verified.

Experimental

Instrumentation

An Agilent 1290 Infinity Binary pump system was connected to a Dual Jet Stream source of an Accurate Mass 6540 Q-TOF system. The source conditions used for the 6540 Q-TOF MS system are shown in Table 1. A low fragmentor voltage of 120 V was used for MS and (auto) MS/MS experiments, while a fragmentation voltage of 250 V was used for pseudo MS^3 experiments.

Software

Agilent MassHunter Qualitative Analysis Software (Version B.07.00), Agilent Personal Compound and Database Library (PCDL) Manager (Version B.07.00), Molecular Structure Correlator (MSC) Software (Version B.07.00). All structures were drawn using ACD/ChemSketch, and fragmentation pathways confirmed using ACD/MS Fragmentor 2012 software (ACD Labs, Toronto, Canada).

Table 1. Conditions used for the Agilent 6540 Q-TOF MS system.

Parameter	Value
LC column	Agilent ZORBAX Eclipse Plus Phenyl-Hexyl RRHD, 1.8 µm 3.0 × 50 mm, (p/n 959757-312)
Gas temperature	175 °C
Drying gas (nitrogen)	9 L/min
Nebulizer gas (nitrogen)	40 psi
Sheath gas temperature	200 °C
Sheath gas flow	9 L/min
Capillary voltage	2,500 V
Nozzle voltage	500 V
Skimmer voltage	40 V
Oct 1 RF Vpp	700 V

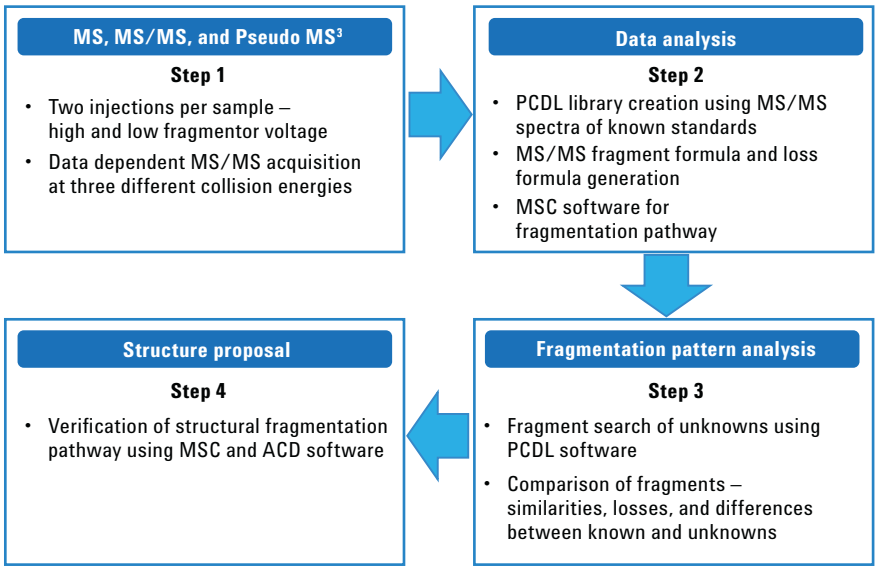

Figure 1. Workflow used in the structure elucidation of amlodipine impurities.

Known API and impurity standards

Amlodipine, Impurity A, Impurity B, Impurity C, Impurity D, Impurity E, Impurity F, Impurity G, Impurity H

Unknown sample

Amlodipine in-process sample obtained from Anant Pharmaceuticals.

Sample preparation

All compounds were dissolved in 100 % methanol and injected at a concentration of ~100 ng/mL.

Table 2 shows the experimental details.

Table 2. Experimental details.

Parameter	Value
Column temperature	40 °C
Autosampler temperature	6 °C
Injection volume	5 µL
Flow rate	0.5 mL/min
Mobile phase	A) 10 mM Ammonium bicarbonate in water B) Methanol
Gradient	Time %B 2.0 40 7.0 55 10.0 55 19.0 95 24.0 95 24.1 40
Run time	30 minutes
Mass range	50–1,200 *m/z*
MS scan rate	3 spectra/sec
MS/MS scan rate	1 spectra/sec
Collision energy	Fixed: 10, 20, 40 eV

Results and Discussion

Analysis of standard compounds

Data-dependent MS/MS spectra of amlodipine standard (99 % pure) were acquired at low and high source voltage as shown in Figures 2 and 3, respectively. At low source voltage, data-dependent MS/MS spectra were acquired for the protonated molecular ion MH+ at *m/z* 409.1525 (Figure 2B), and for the fragment ions at *m/z* 294.0892 (Figure 2C) and *m/z* 238.0628 (Figure 2D). The MS/MS spectra of *m/z* 409.1525 yielded major fragment ions at *m/z* 377.1259, 294.0892, and 238.0628. The results show that the response for amlodipine is very good using electrospray ionization conditions, and that the compound is sufficiently labile to give significant fragmentation with low source voltage conditions. Under pseudo MS^3 conditions, (Figure 3A), MS/MS spectra were acquired for amlodipine fragment *m/z* 377.1265 (Figure 3B). The formulae of each fragment were generated using the Generate Formulas feature within Agilent MassHunter Qualitative Analysis Software.

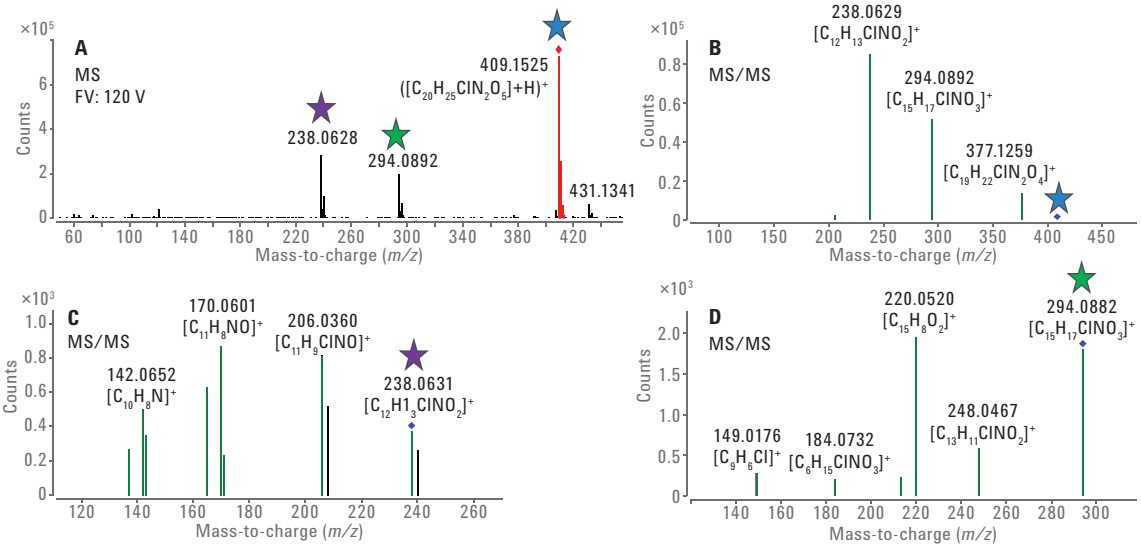

Figure 2. (A) MS spectra at low source voltage, extracted from data-dependent MS/MS acquisition (B) MS/MS spectra of amlodipine (MH^+ at *m/z* 409.1525), (C) MS/MS spectra of the fragment at *m/z* 238.0628 and (D) MS/MS spectra of the fragment at *m/z* 294.0892.

Formula and loss formula determination

Table 3 shows the MS/MS results at low source voltage for amlodipine standard. The *m/z*, proposed formula, mass difference (ppm) between experimental and theoretical formula of calculated mass, loss mass, and loss formula for each MS/MS fragment is shown. The loss formula is calculated from the chemical formula of amlodipine ($C_{20}H_{25}ClN_2O_5$). The fragment at *m/z* 208.0604 has two possible formulae: one with, and one without chlorine. Since no characteristic chlorine isotope pattern was observed, it is concluded that the formula $C_{10}H_{10}NO_4$ (0.4 ppm error) is the correct one. A similar list was generated for all known standard samples. Each spectrum was then exported to PCDL software to create a custom library.

Molecular Structure Correlator software

The MSC software matches accurate mass MS/MS fragment ions with one or more theoretical fragments from the proposed structure. In this work, the ChemSpider (Royal Society of Chemistry) database was searched for possible structures that correspond to the experimental MS/MS data. Figure 4 shows the MSC output, and Figure 5 shows the possible fragmentation sites for amlodipine, Impurity H, and Impurity E standards.

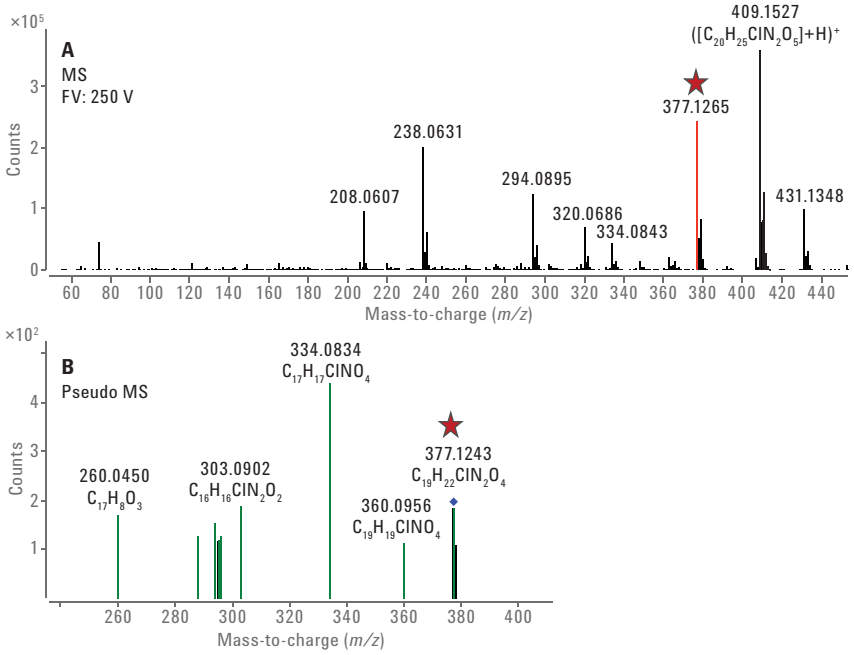

Figure 3. MS spectra at high source voltage, extracted from data-dependent MS/MS acquisition (A), and pseudo MS^3 spectra (B).

Table 3. MS/MS spectra formula details for amlodipine $C_{20}H_{25}ClN_2O_5$. The *m/z* values without formula assignment indicates that no formula could be proposed that matches to the precursor formula within 5 ppm.

MS/MS Formula Details: Cpd 21: C20 H25 Cl N2 O5 C20 H25 Cl N2 O5

m/z	Formula	Diff (ppm)	Loss Mass	Loss Formula
238.0628	C12 H13 Cl N O2	0.52	171.0895	C8 H13 N O3
294.0891	C15 H17 Cl N O3	0.17	115.0633	C5 H9 N O2
240.0601				
208.0604	C10 H10 N O4	0.4	201.092	C10 H16 Cl N O
208.0604	C7 H13 Cl N2 O3	2.74	201.0916	C13 H13 O2
377.1259	C19 H22 Cl N2 O4	0.95	32.0262	C H4 O
206.0365	C11 H9 Cl N O	0.92	203.1158	C9 H17 N O4
296.0864				
220.0522	C12 H11 Cl N O	0.97	189.1001	C8 H15 N O4
170.0596	C11 H8 N O	2.83	239.0924	C9 H18 Cl N O4
176.0339	C9 H6 N O3	1.95	233.1183	C11 H20 Cl N O2
379.1237				
165.0102	C9 H6 Cl O	-0.02	244.1423	C11 H20 N2 O4
334.0838	C17 H17 Cl N O4	0.87	75.0684	C3 H9 N O
208.0333				
248.0467	C13 H11 Cl N O2	2.43	161.1052	C7 H15 N O3
260.047	C14 H11 Cl N O2	0.9	149.1052	C6 H15 N O3
142.0652	C10 H8 N	-0.41	267.0874	C10 H18 Cl N O5
320.0687	C16 H15 Cl N O4	-0.75	89.0841	C4 H11 N O
149.0148	C9 H6 Cl	3.31	260.1372	C11 H20 N2 O5
222.0495				
232.0522	C13 H11 Cl N O	0.73	177.1001	C7 H15 N O4
303.0886	C16 H16 Cl N2 O2	2.97	106.063	C4 H10 O3
143.0724	C10 H9 N	4.07	266.0795	C10 H17 Cl N O5
137.0149	C8 H6 Cl	2.6	272.1372	C12 H20 N2 O5
184.0753	C12 H10 N O	2.05	225.0768	C8 H16 Cl N O4

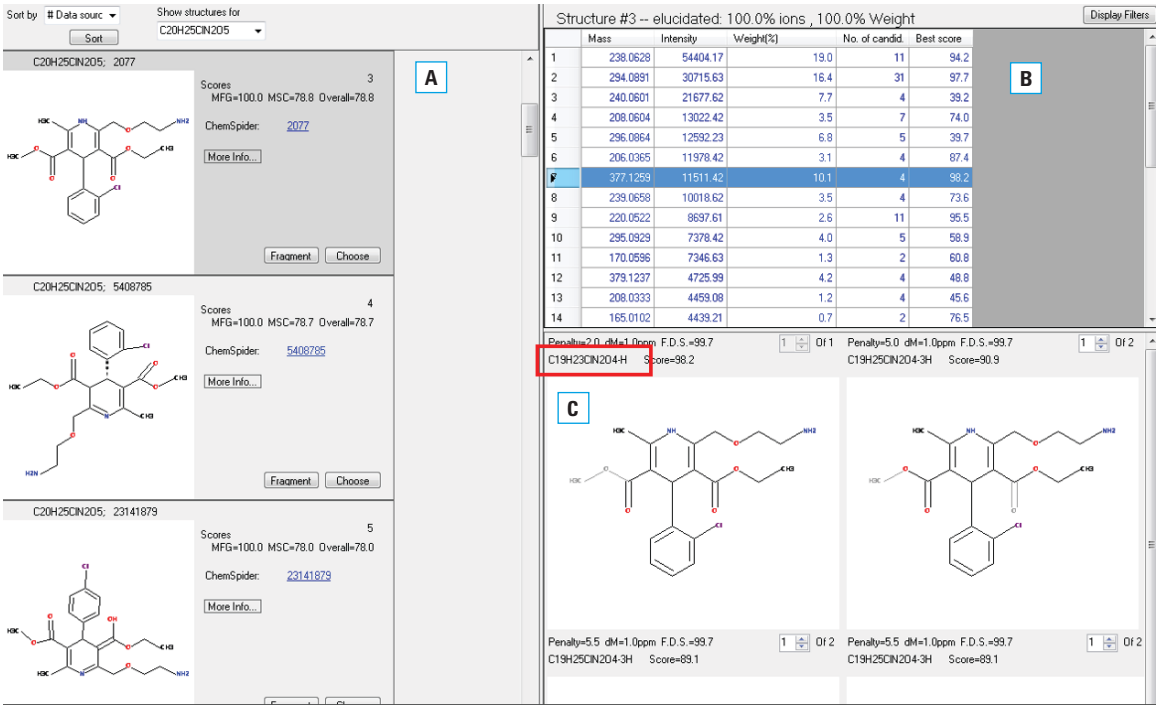

Figure 4. MSC software showing possible fragmentation sites; the red box indicates the formula generated. A) Possible structures matching the experimental MS/MS results. B) Experimental MS/MS results and matching scores. C) Proposed fragmentation sites.

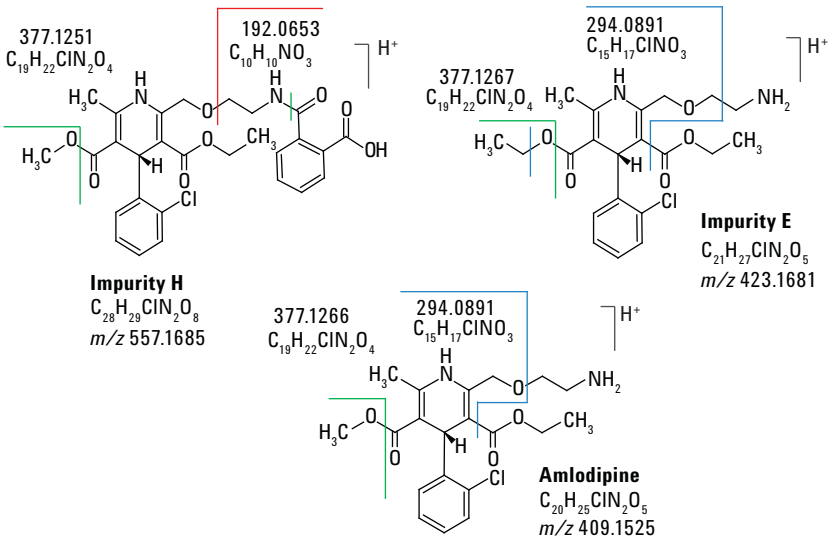

Figure 5. Possible fragmentation sites of a known standard proposed by MSC software.

Unknown sample analysis

Accurate mass MS analysis

The unknown sample analysis in MS mode revealed nine components (Figure 6) using the molecular feature extraction algorithm. Table 4 gives the formula of each of these compounds, along with the mass error.

Fragment matching and proposed structures

The structural elucidation of the compound at *m/z* 630.2215 (Peak 4, Figure 6) is shown. PCDL software enables fragment matching of unknowns with fragments in the library. The MS/MS fragment at *m/z* 294.0880, of Peak 4 was found in Impurity E, while the ion at *m/z* 192.0653 was found in Impurity H (Figure 7). In addition, the pseudo MS^3 spectra of the ion at *m/z* 294.0880 showed a similar fragmentation pattern to the ion at *m/z* 294.0880 in Impurity H and E (data not shown).

Fragmentation pathways

The Peak 4 substructure was proposed based on the core structure of the ion at *m/z* 294 and the fragment ion at *m/z* 192 (right hand side of the molecule). Figure 8 shows the fragmentation pathway for the ion at *m/z* 630.2215. The proposed structures and fragmentation were confirmed using the ACD software fragmentor feature. Here, the proposed structure was drawn using ACD software and fragmented *in silico* by selecting ESI fragmentation. The results yielded the fragments *m/z* 294.089 and *m/z* 192.0654, and are consistent with expected fragmentation mechanisms.

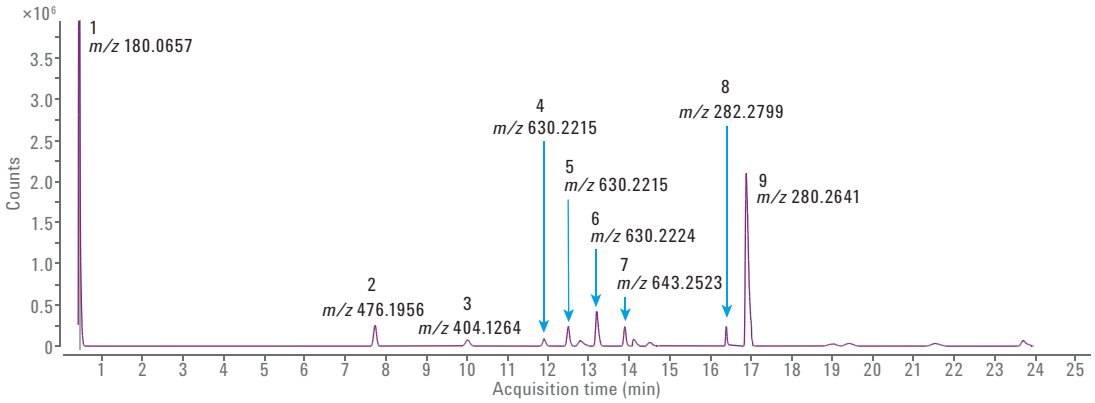

Figure 6. TIC (background subtracted) of the in-process samples showing nine compounds and their measured accurate masses.

Table 4. Nine compounds detected from the unknown impurity samples and their calculated accurate mass errors.

Peak label	Peak 1 RT = 0.47	Peak 2 RT = 7.7	Peak 3 RT = 9.9	Peak 4,5,6 RT = 11.9	Peak 7 RT = 13.9	Peak 8 RT = 16.4	Peak 9 RT = 16.9
m/z	180.0657	476.1956	404.1264	630.2215	643.2523	280.2641	282.2799
Calculated mass	179.0586	475.1886	403.1196	629.2147	642.2453	279.2574	281.2729
Mass formula	$C_9H_9NO_3$	$C_{24}H_{30}ClN_3O_5$	$C_{21}H_{22}ClNO_5$	$C_{31}H_{36}ClN_3O_9$	$C_{32}H_{39}ClN_4O_8$	$C_{18}H_{33}NO$	$C_{18}H_{35}NO$
Mass accuracy (mDa)	0.32	1.23	0.93	0.73	0.33	1.23	1.01
Mass accuracy (ppm)	1.81	2.59	2.31	1.16	0.51	4.42	3.6

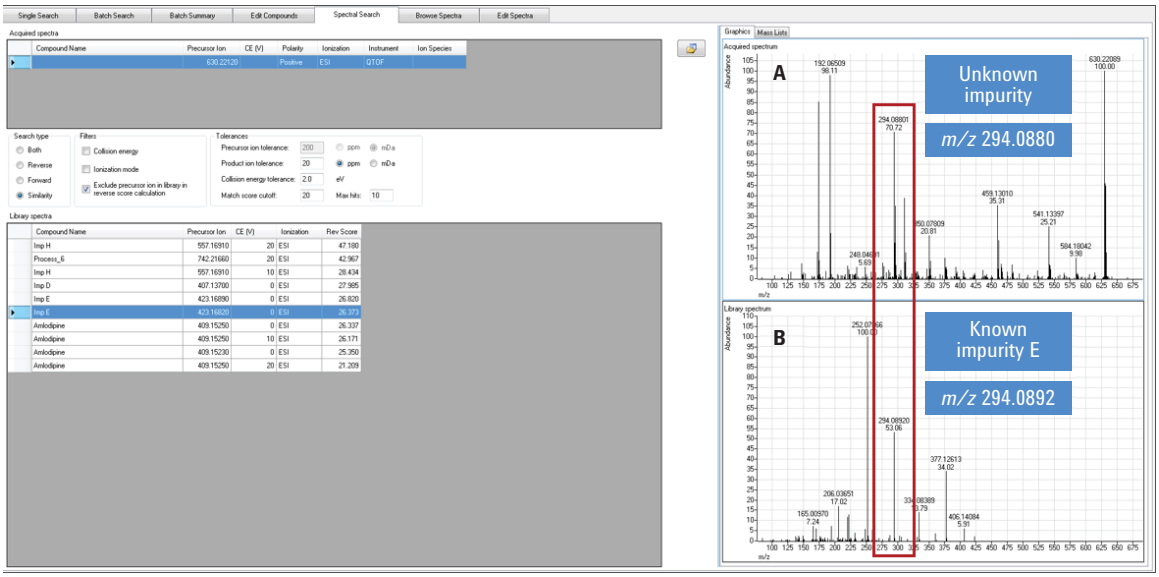

Figure 7. Fragment matching of unknown spectra with library spectra of the PCDL. The MS/MS spectra of *m/z* 630.2215, Peak 4 impurity (A1) matched with the library fragment *m/z* 294.0892 of impurity E (B).

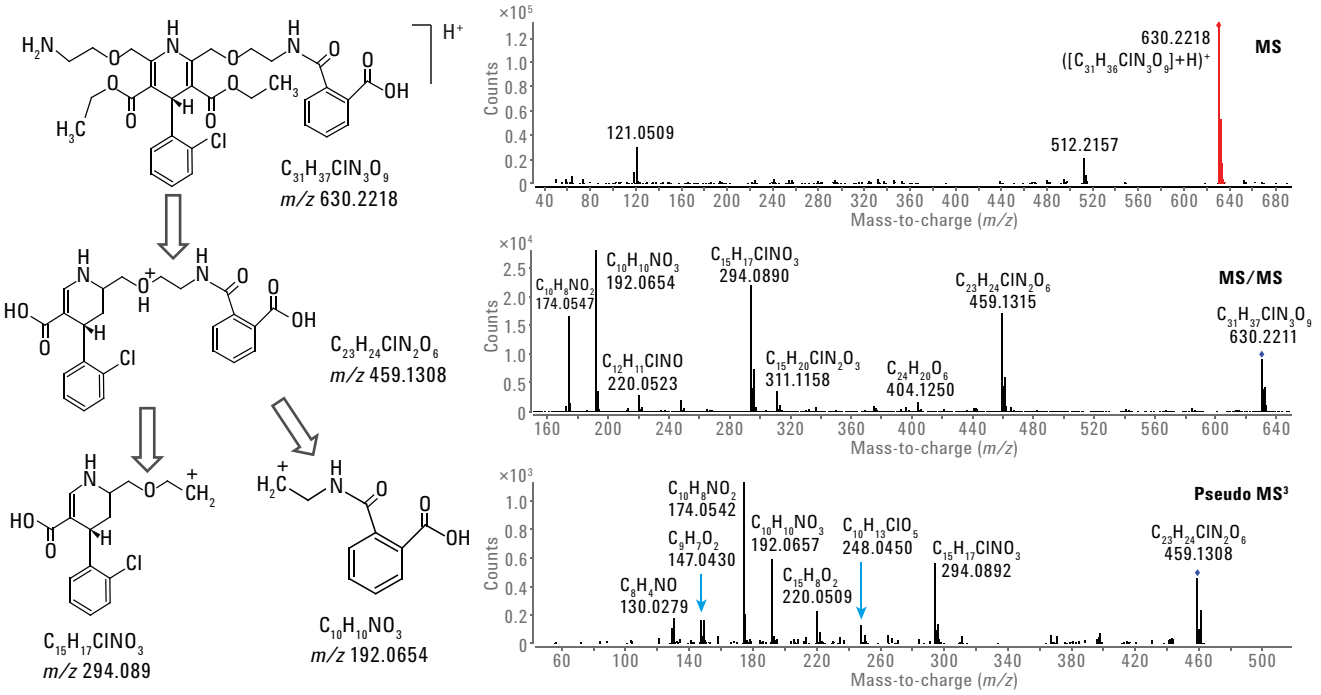

Figure 8. Proposed structure and fragmentation pathway of the unknown ion at *m/z* 630.2218 (Peak 4). The pseudo MS^3 of *m/z* 459.1308 revealed that it is an intermediary for fragment ions at *m/z* 294.089 and *m/z* 192.0654.

The MSC software also proposed the same fragmentation scheme for the proposed structure. Using this approach along with the knowledge of the reaction ingredients, the structures of other impurities were proposed (Figure 9).

Conclusion

This work describes the structure elucidation of amlodipine impurities. As a result of this analysis, nine impurity compounds were separated and identified. These have a range of different chemical structures, and were formed during the commercial synthesis process. Available standards and in-process impurity samples were analyzed by high-resolution accurate mass LC-MS and LC-MS/MS using an Agilent 1290 Infinity LC system Q-TOF MS, MS/MS, and pseudo MS^3 modes. The fragmentation patterns of known compounds were determined and stored in the PCDL software to enable fragment searching within the custom database. The fragmentation sites and pathways of known standard compounds were determined using Molecular Structure Correlator software to confirm the cleavage sites and to propose structures and substructures for the unknowns. Thus, the combination of intelligent software and high-resolution accurate mass data enabled the structures of several impurities to be determined.

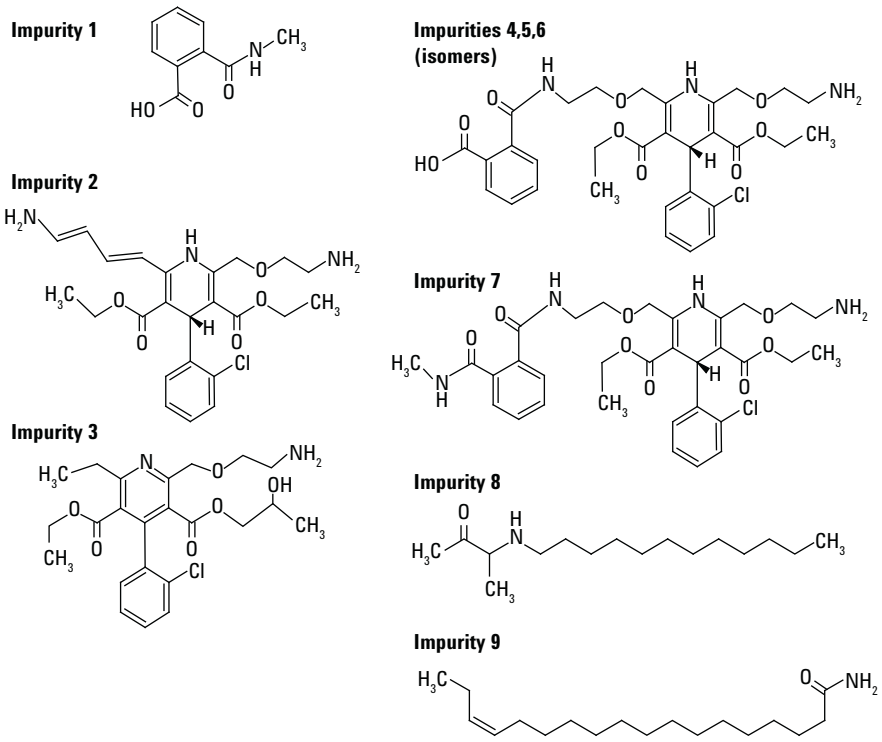

Figure 9. Proposed structures of impurities in the unknown sample.

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Published in the USA, November 1, 2015
5991-6411EN

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