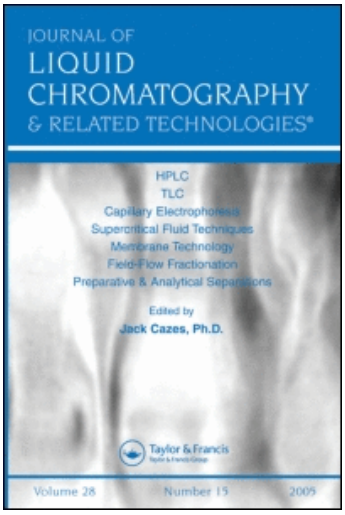


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# Identification and Control of Impurities for Drug Substance Development using LC/MS and GC/MS

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# Identification and Control of Impurities for Drug Substance Development using LC/MS and GC/MS

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**Abstract:** Identification and control of impurities for drug substances is a critical task in pharmaceutical process development for quality and safety. The most commonly used analytical technique for impurity analysis in drug substances and drug products is undoubtedly a chromatographic method, namely high performance liquid chromatography (HPLC). Impurity profiling is typically performed by HPLC and impurities are further tested for identification and confirmation by other techniques. Several case studies are presented in this paper to report the identification of unknown impurities employing chromatographic techniques interfaced with mass spectrometry. The task of unknown identification was facilitated by complementary methodologies including tandem mass spectrometry (MS/MS), high resolution mass spectrometry (HRMS), preparative HPLC and NMR. Upon identification of the impurity, the impurity formation was monitored and controlled throughout the synthesis. Three case studies are described where unknown process impurities were analyzed for identification using LC/MS and GC/MS methodologies. It is demonstrated that identification of the unknown impurity enabled chemists to pinpoint the chemical step of impurity generation, aiding the effort to reduce or even eliminate the impurity in the drug substances.

**Keywords:** Control of impurities, Drug development, GC/MS, LC/MS

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## INTRODUCTION

Impurity profiling is one of the most critical analytical tasks during the development of drug substances (also known as active pharmaceutical ingredients, API). The level of impurities is tightly controlled by regulatory agencies for toxicological assessment and clinical studies. ICH guideline Q3A(R) requires that organic impurities at or above 0.1% (or 1.0 mg total daily intake, whichever is lower) should be identified for drug substance with maximum daily dose of less than 2 g/day.[1] The most established analytical method for impurity profiling is indisputably high performance liquid chromatography (HPLC) with UV detection. The organic impurities are usually determined by HPLC/UV first and further analyzed by other analytical techniques including LC/MS, MS/MS, HRMS, preparative LC, and NMR (nuclear magnetic resonance). With the advent of atmospheric pressure ionization methods such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) enabling a smooth transition of samples from the liquid phase (HPLC) to the gas phase (MS), LC/MS has become a prominent analytical technique for both quantitative and qualitative purposes in pharmaceutical research and development.[2]

Identification and tracking of organic impurities using LC/MS related technologies for drug substance[3–12] and drug product[13–16] development is well documented in the literature. Most of the reported cases employed multi-disciplinary approaches in order to elucidate the impurity structures, namely, tandem mass spectrometry ($MS^n$), high resolution mass spectrometry (HRMS), LC/UV, LC/MS, LC/NMR, NMR and preparative LC. Quadrupole-based mass spectrometers (single quadrupole and triple quadrupole MS) are by far the most widely used MS types. Single quadrupole MS can provide molecular ion information and in certain cases fragmentation data through in-source collision induced dissociation (CID). In comparison, triple quadrupole MS is useful for acquisition of $MS^2$ tandem mass spectral data. It has been reported that trace level impurities were identified using ion trap multistage tandem mass spectrometry followed by preparative LC for confirmation by NMR.[3,10] Ion trap-based mass spectrometry provides $MS^n$ fragmentation pathways, compared to quadrupole-based mass spectrometry which offers $MS^2$ stages, rendering in-depth structural information for impurity identification.[2] High resolution mass spectral data can add another dimension to the analytical information for the determination of impurity by providing elemental compositions.[11,14,15] The combination of the multistage tandem mass spectrometry and accurate mass measurement gives an extremely powerful analytical tool, FT-ICR-MS (Fourier Transform Ion Cyclotron Resonance Mass Spectrometry). This device

is capable of providing elemental compositions of the molecular and fragment ions for the n-th order of tandem mass spectral data. Several groups reported the use of FT-ICR-MS for the identification and confirmation of unknown impurities.[8–10] The process impurities are expected to have structural similarity to the drug substance. Therefore, the mass spectral fragmentation pattern of the process impurities were compared to that of the drug substance, and impurity structures were postulated in the reports. The identity was subsequently confirmed by NMR experiments on isolated impurities, or in certain cases the postulated compound was synthesized for unequivocal confirmation.

Isolation of the impurity via column chromatography or preparative HPLC is a labor-intensive and time-consuming task, and therefore it is avoided whenever possible. However, there were reported cases where the unexpected toxicity and color of the drug substance made the fractionation of the impurity inevitable. The impurity responsible for the unexpected color of the drug substance was much less than 0.1%, but the impurity had to be identified to understand the origin of formation and to reduce or eliminate the impurity.[4] An unexpected toxic response during an animal toxicological study from a drug substance batch was observed, and the batch was fractionated to pinpoint the impurity, though the level of the impurity was lower than 0.1%.[11] In both cases, preparative HPLC was used to fractionate and enrich the impurities.

Most drug substances are too polar or thermally labile to be subject to a gas chromatographic analysis, which requires vaporization of the samples into the gas phase. However, for analysis of raw material and intermediates, gas chromatography offers several advantages including high separation efficiency, wide dynamic range, and various compatible detectors such as FID (flame ionization detection), ECD (electron capture detection), TCD (thermal conductivity detection), and MS. Especially GC/MS is a powerful analytical technique for identification of unknowns due to the availability of vast and accessible spectral libraries.

In this report, three case studies of unknown process impurities are presented. The impurities were first detected by HPLC/UV and GC/FID, and they were subject to LC/MS and GC/MS analyses to obtain molecular ion information. Further analyses such as MS/MS, HRMS, preparative HPLC, NMR, and synthesis of the authentic compound were performed to elucidate the impurity structures. The knowledge obtained helped to understand impurity formation and was used to improve the quality of the drug substances.

## EXPERIMENTAL

### Reagents

HPLC grade water and acetonitrile were obtained from EMD Chemicals (San Diego, CA, USA). Formic acid was purchased from Fluka (St. Louis, MO, USA), and perchloric acid was obtained from Sigma Aldrich (St. Louis, MO, USA). All reagents were used as received.

### Instrumentation

Agilent (Santa Clara, CA, USA) 1100 HPLC systems with ChemStation software were employed for liquid chromatography work. A typical HPLC system consisted of a degasser, a binary pump, an autosampler, an ALS thermostat, a column compartment, and a diode array detector (DAD). GC/FID and GC/MS data were obtained using Agilent G6890N with FID (flame ionization detection) or MSD (mass selective detection) with ChemStation software. A Micromass/Waters (Milford, MA, USA) Quattro Ultima triple quadrupole mass spectrometer with MassLynx control was used for tandem mass spectrometric data generation. An ionization mode of positive electrospray was used with the following parameters: capillary voltage 3.5 kV, cone voltage 20–35 V, drying gas nitrogen, collision gas argon, and collision cell pressure $1.0 \times 10^{-3}$ mbar. High resolution mass spectral data were obtained using an Agilent LC/MSD TOF mass spectrometer in a positive electrospray ionization mode with the following parameters: capillary voltage 3.0 kV, drying nitrogen gas temperature 350°C, and fragmentor 70–150 V. The samples were introduced to the mass spectrometer via flow injection without a chromatographic separation. Impurity purification by preparative LC was performed using a Shimadzu (Kyoto, Japan) system consisting of a SIL-10AP auto-injector, two LC-8A pumps, SPD-10A VP UV-VIS, SCL-10A VP system controller, and FRC-10A fraction collectors with Discovery VP software.

### Case Study A

#### Analytical HPLC Conditions

The column used for analytical HPLC experiments was an Agilent Zorbax SB-CN 4.6 mm × 150 mm, with particle size of 3.5 um. Mobile phase *A* was water with 0.5% (v/v) perchloric acid, and mobile phase *B* was acetonitrile. The flow rate was 1.5 mL/min with the column

temperature maintained at 25°C. Gradient elution profile was to hold at 0%B for 3 min, linear gradient to 40%B at 4 min, hold at 40%B until 12 min, linear gradient to 60%B at 17 min, to 100%B at 18 min, and hold at 100%B until 20 min. The sample diluent was methanol and injection volume was 5 μL. UV detection wavelength was 220 nm. The perchloric acid used in the mobile phase was replaced with formic acid for acquisition of mass spectral data.

### Preparative HPLC Conditions



For preparative purification of the impurity, a Waters XBridge Prep C18 OBD (19 mm ID × 150 mm L, with 5 μm particle size) was used. The mobile phase *A* was water and the mobile phase *B* was acetonitrile without any modifier. The use of modifier in the preparative LC was intentionally avoided whenever possible to prevent any decomposition of the collected samples, even though it meant a slight loss in the chromatographic resolution and peak shape. The acidic or basic modifier used in the LC mobile phase could become concentrated during the solvent evaporation step, and cause decomposition of the collected fractions.[5] The flow rate was 10 mL/min with a gradient elution profile from 10%B to 37%B in 20 min, and constant at 37%B until 50 minutes. The UV wavelength was at 220 nm and the column was held at an ambient temperature. The sample diluent used was methanol. The solvents were evaporated from the fractions using a rotary evaporator.

### GC/FID Conditions to Monitor the Intermediate B

The GC column used was DB1701, 30 m length, 320 μm ID, with film thickness of 1.0 μm. The carrier gas was helium with a constant flow rate of 1.5 mL/min. The inlet temperature was 250°C with a split of 30:1. The oven temperature profile was to hold at 80°C for 0.5 min, to ramp to 220°C at a rate of 15°C/min, and to hold at 220°C for 1 minute. The FID temperature was 300°C.

## Case Study B

A Waters XBridge C18 column (4.6 mm × 75 mm, with 2.5 μm particle size) was used and water (mobile phase *A*) and acetonitrile (mobile phase *B*) with 0.1% formic acid were employed as mobile phases. The flow rate was 2.0 mL/min with a gradient of 20%B to 90%B in 10 minutes. The column temperature was kept at 40°C.

### Case Study C

The GC column was Agilent DB1701, 30 m × 0.32 mm ID, with 1 μm film thickness. Inlet temperature was at 200°C with helium as a carrier gas, constant pressure at 10 psi, and split of 25:1. The GC oven temperature profile was to hold at 35°C for 7 minutes, ramp to 80°C at 7°C/min, ramp to 180°C at 15°C/min, then hold at 180°C for 2 minutes. The FID temperature was 250°C.

Additional experimental conditions are described as needed in the Results and Discussion section for each example discussed.

## RESULTS AND DISCUSSION

### Case Study A

An HPLC method was developed for reaction monitoring of a fixed synthetic route for a development compound. Figure 1(a) shows an

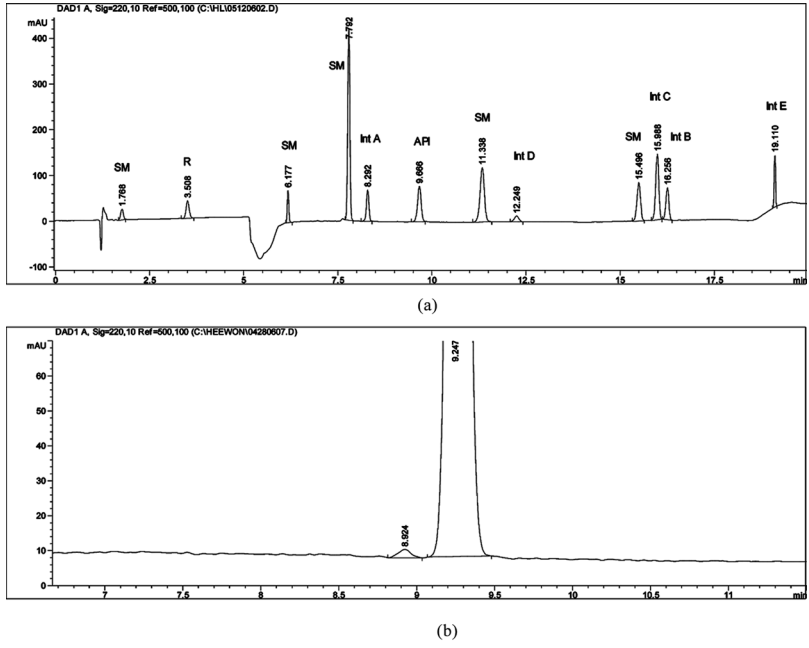



***Figure 1.*** Case Study A: (a) Chromatographic separation of starting materials (SM), reagents (R), intermediates (Int), and API. (b) The HPLC chromatogram showing the impurity contained in the first batch of the drug substance.

HPLC chromatogram of the starting materials, reagents, intermediates, and the drug substance (i.e., API). This chromatogram was obtained from an artificial mixture of the involved compounds in order to illustrate the chromatographic separation. The intermediates and API were indicated on the chromatogram. This HPLC method was successfully utilized during the synthetic steps. The synthetic route involved a linear synthesis (intermediates A → B → C → D → E) for the production of API. This method was employed to profile impurities in the first batch of the drug substance. The batch did not have any detectible amount of intermediates, but there was an impurity in the level of 0.6% eluting right before the product shown in Figure 1(b).



In order to identify this unknown impurity, LC/MS experiments were performed. The experimental conditions of the HPLC method were transferred to LC/MS, except for the mobile phase composition. The 0.5% perchloric acid in the mobile phase A of the original HPLC method was substituted with 0.1% formic acid for LC/MS experiments for modifier volatility. This substitution did not affect the selectivity of the impurity from the drug substance considerably.

Figure 2(a) shows the total ion chromatograms (TIC) of the drug substance and the crystallization mother liquor. The retention time of the drug substance was 7.8 minutes and that of the impurity was 7.5 minutes. The mass spectra show the molecular ion for the drug substance and the impurity in Figure 2(b). The molecular ion of the desired product was observed at m/z 396, and the impurity showed an ion at m/z 378 with a mass difference of 18 amu. For tandem mass spectrometric experiments the mother liquor obtained from crystallization was utilized in order to obtain stronger signal intensity of the impurity.

One of the most widely used tandem mass spectrometric techniques using a triple quadrupole MS is the daughter ion (also known as fragment ion) scan. In this scan mode, the first quadrupole is set to pass only the ion of interest, usually the molecular ion. The second quadrupole is used as a collision cell with a controlled amount of collision gas, typically argon. The selected ion from the first quadrupole undergoes collision induced dissociation (CID) in the second quadrupole. The fragment (daughter) ions generated in the second quadrupole are then mass-resolved in the third quadrupole, generating structure-specific fragmentation information.

The MS/MS experiments were performed to obtain daughter ion spectra of the drug substance molecular ion at m/z 396. The fragmentation spectra were acquired at different collision energies (10, 20, 30, 40, 50 and 60 V) in order to cover various fragmentation patterns. The two representative spectra at different collision energies are shown in Figure 3(a). As expected, the molecular ion was still present and a few fragment ions were observed at low collision energies; and at

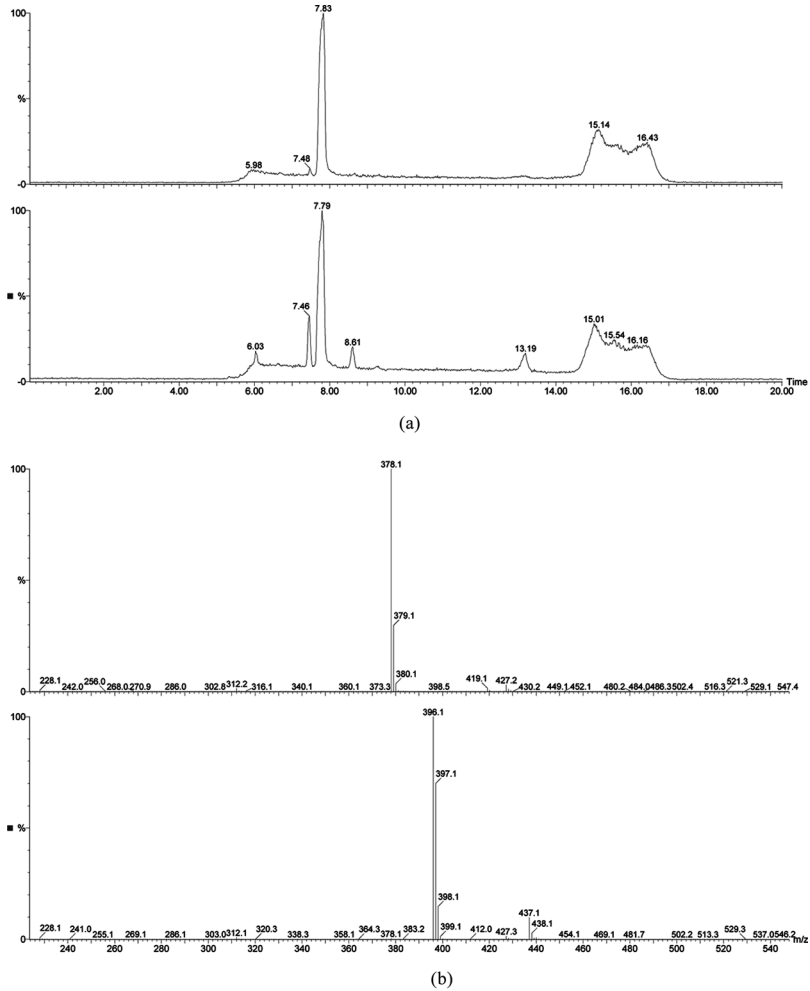



***Figure 2.*** Case Study A: (a) Total ion chromatogram (TIC) of the drug substance (above) and the mother liquor (below). The retention time of the drug substance is 7.8 minutes and that of the impurity is 7.5 minutes. (b) The mass spectra show the molecular ion of the impurity at m/z 378 (above) and the drug substance at m/z 396 (below).

high collision energies the molecular ion disappeared and more extensive and smaller fragment ions were observed. The daughter ion scan of the impurity at m/z 378 showed a similar trend in Figure 3(b).

The illustrative structure of the drug substance is shown below with the key fragments observed in the daughter ion scan data. The mass

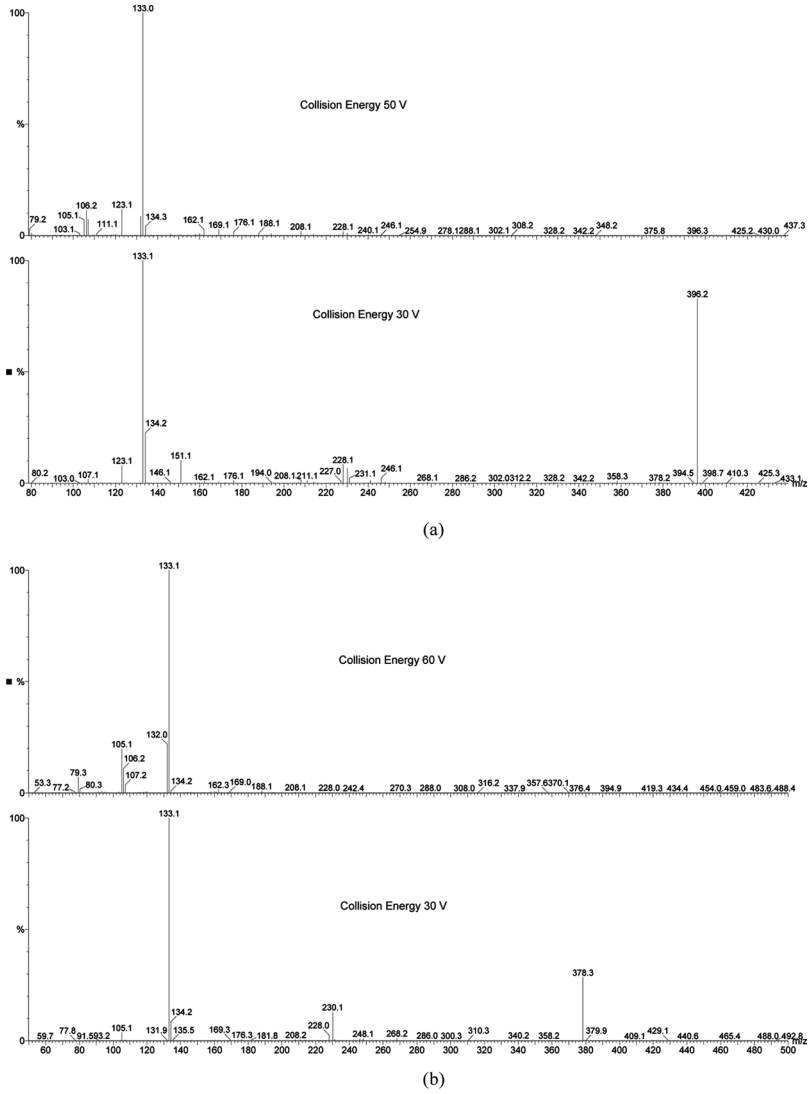
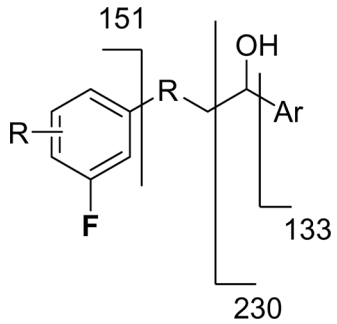



***Figure 3.*** Case Study A: Fragment ion mass spectra at two difference collision energies are shown for the drug substance at m/z 396 (a) and for the impurity at m/z 378 (b).

difference of 18 between the drug substance and the impurity could be the loss of hydroxyl group ($-H_2O$) or the loss of the fluorine atom replaced with a hydrogen ($-19$ amu for fluorine $+1$ amu for hydrogen $= -18$ amu). Both the drug substance and the impurity had the fragment

ions at m/z 133 and m/z 230, indicating that the right hand side of the molecule was intact including hydroxyl functional group. However, the fragment ion at m/z 151 was present only in the drug substance daughter ion scan, noticeably absent in the daughter ion spectra of the impurity. Therefore, the difference between the drug substance and the impurity must have occurred in the left hand side of the molecule. Based on the data, the impurity was tentatively identified as the des-F (loss or removal of fluorine) of the drug substance. The ion corresponding to the m/z 151 of the des-F impurity would be m/z 133 (m/z 151–18) that happened to coincide with the m/z 133 from the right hand side of the molecule.






Structure of the drug substance with observed fragments for Case Study A.

The impurity was isolated using a Shimadzu preparative LC system in order to confirm the identity. The retention time of the impurity was 44 minutes and that of the drug substance was 47 minutes under the experimental conditions. The fractions collected were subject to high resolution mass spectrometry (HRMS) using an Agilent 1100 LC and an Agilent TOF (time-of-flight) mass spectrometer. The HRMS data showed the correct elemental formula for the drug substance with an error of 0.1212 ppm. The elemental formula based on the HRMS data of the impurity fraction matched that of the des-F impurity with an error of 2.3104 ppm.

The proposed des-F impurity was synthesized to confirm the identity unequivocally. The NMR data were acquired for both the impurity collected by preparative LC and the synthesized des-F API, and the NMR experiments confirmed that they were identical. In an effort to control the des-F impurity in the drug substance, corresponding des-F compounds for intermediates A, B, C, and D along the synthetic route were synthesized. The des-F intermediates A, C, and D were separated under the existing HPLC conditions as shown in Figure 4(a). The separation of intermediate B and des-F intermediate B was not ideal

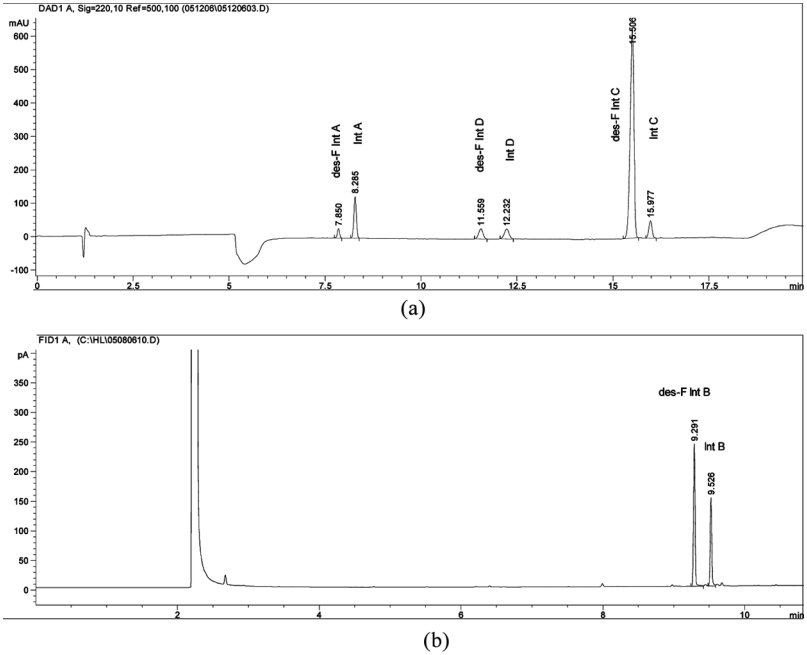



***Figure 4.*** Case Study A: (a) HPLC chromatographic separation of the des-F intermediates A, C, and D from the corresponding intermediates for impurity control. (b) GC/FID method showing the separation of the des-F intermediate B from the intermediate B for impurity control.

in HPLC conditions, thus a GC/FID method was developed to follow the intermediate B as shown in Figure 4(b).

The HPLC/UV and GC/FID methods shown in Figure 4 were employed to carefully monitor the formation of des-F impurities along the synthetic pathway. It was found that the des-F impurity was formed from the chemical step of the intermediate A to the intermediate B, where an equimolar amount of lithium diisopropylamide (LDA) was employed. The formation of the des-F impurity was eliminated by slightly undercharging LDA.

In this case study, the structure of the unknown process impurity was proposed through LC/MS/MS and HRMS. The proposed structure was confirmed by preparative LC, synthesis of proposed compound, and NMR experiments. The origin of the impurity formation was pinpointed, and analytical methods were developed to follow and control the impurity formation throughout the synthesis. We were able to optimize the chemical synthesis so that the process did not generate the impurity.

## Case Study B

The drug substance for this project was prepared as a free acid, and subsequently converted to a potassium salt. During the salt formation step a new impurity with an area percent of 2.4% was observed. The molecular ion $[M + H]^+$ for the drug substance was at m/z 642 (Figure 5), with one chlorine indicated by the isotope pattern. The molecular ion of the impurity was at m/z 638, four amu less than the API, clearly missing the isotope pattern of chlorine. As can be seen in the LC/MS data, molecular ions for both the drug substance and the impurity underwent significant in-source collision induced dissociation (CID) generating abundant fragmentation. The major fragment ions observed for the API were m/z 435, 338 and 267, with all three ions showing the pattern of one chlorine. The major fragment ions observed for the impurity were m/z 431, 334, and 263. Interestingly, all three ions were four amu less than the corresponding fragments in the API, and they lacked the chlorine isotope pattern.

In-source CID provided useful fragmentation data for these molecules without the need of a triple quadrupole mass spectrometer. The LC/MS/MS experiments were performed to obtain more detailed fragment ion spectra with a reduced noise level providing higher

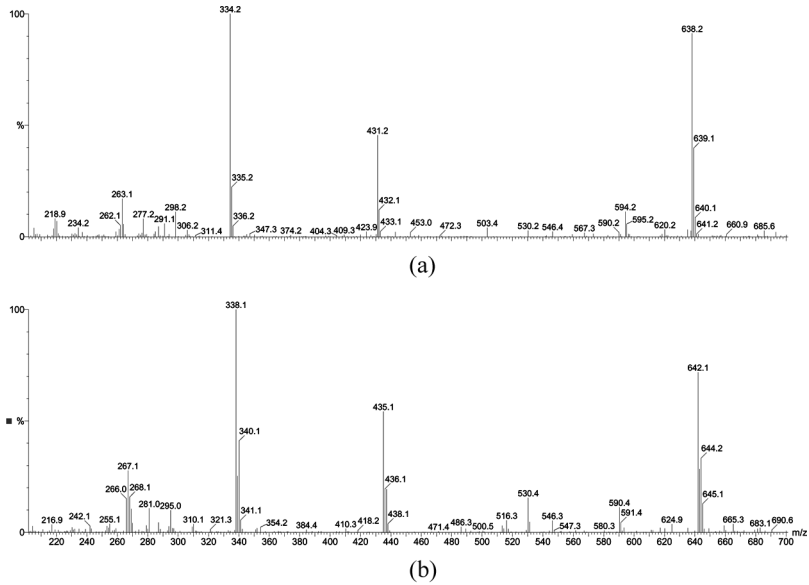
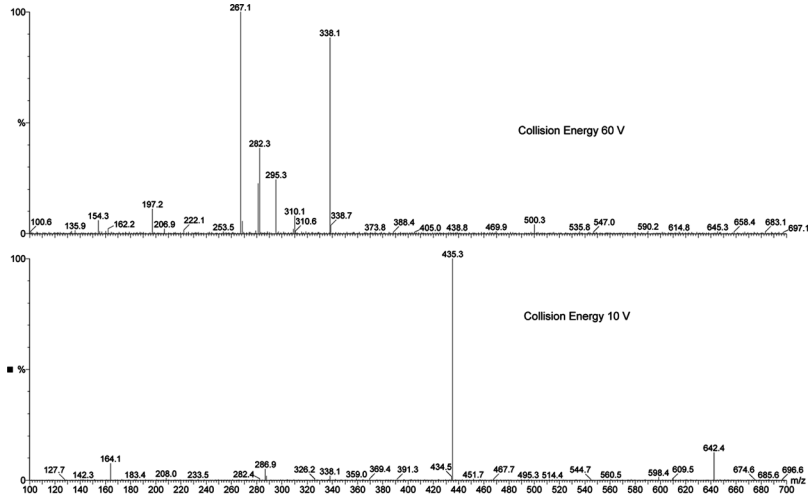
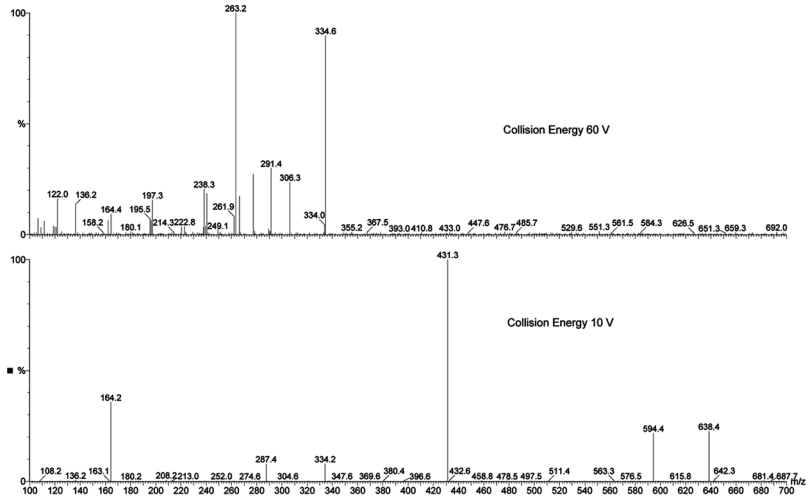



***Figure 5.*** Case Study B: LC/MS spectra of the drug substance (below) and the impurity (above).

confidence. The daughter ion scan was carried out at different collision energies (10, 20, 30, 40, 50, and 60 V) in order to encompass a wide range of fragmentation. Two representative mass spectra at different collision energies are shown in Figure 6(a) for the API and in Figure 6(b) for the impurity. The major ions obtained with LC/MS experiments were still



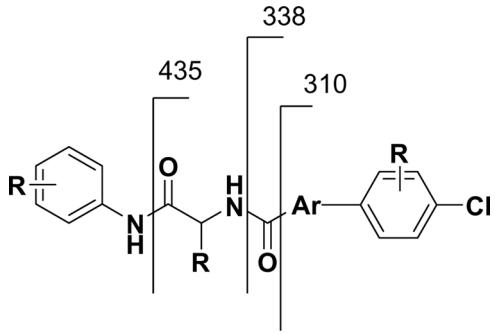

(a)




(b)

***Figure 6.*** Case Study B: Fragment ion mass spectra at two different collision energies (a) for the drug substance at m/z 642 and (b) for the impurity at m/z 638.

the major fragments in the LC/MS/MS data including m/z 435, 338 and 267 for the API; m/z 431, 334, and 263 for the impurity. As the first quadrupole was set at the nominal mass resolution for the LC/MS/MS experiments, the chlorine isotope was filtered out, therefore, missing the isotope pattern information. The structure of the API with key fragments observed is shown below.




Structure of the drug substance with observed fragments for Case Study B.



There were two obvious pieces of information from the LC/MS and LC/MS/MS data. The first information was that the impurity was missing the chlorine, inferred from the molecular ion as well as the fragment ion isotope patterns. The second information was that all three major corresponding fragments of the impurity were four amu less than that of the API, indicating that the modification occurred on the right hand side of the molecule within the fragment structure of m/z 310. Since the molecule had to lose the chlorine, the most straightforward speculation was that "something" replaced the chlorine on the API. The nominal mass of the major isotope of chlorine is 35, therefore, in order to make the impurity to be 4 amu less than the API, "something" had to weigh 31 amu. The proposed impurity structure based on the information was that the chlorine was replaced with a methoxy ($-OCH_3$, 31 amu = 16 amu for O + 15 amu for $CH_3$) group. This made perfect sense as the salt formation was carried out with methanol as the solvent. The proposed impurity structure was further supported by HRMS experiments with an error of 2.5237 ppm. The salt formation step was successfully optimized with regard to the temperature, reagent addition rate, and reagent addition time in order to reduce the impurity formation.

## Case Study C

Residual solvent analysis by GC is a routine analytical test performed for manufacturing of intermediates and drug substances. A GC/FID method including commonly used solvents was developed for this purpose and the conditions are described in the experimental section. One of the process intermediates was analyzed to quantify the residual amount of tetrahydrofuran (THF). The GC/FID data in Figure 7(a) showed a peak corresponding to THF at 6.9 minutes. There was a significant amount of an unexpected impurity present in the intermediate at a retention time of 13.4 minutes. GC/MS experiments were performed to acquire mass spectral data for the impurity. The ionization mode was positive electron impact (EI) ionization at 70 eV. Unlike electrospray ionization (ESI) which generally produces $[M + H]^+$ ions, electron impact (EI) ionization produces $M^{+\cdot}$ radical ions. Electron impact ionization is a "hard" ionization method producing significant

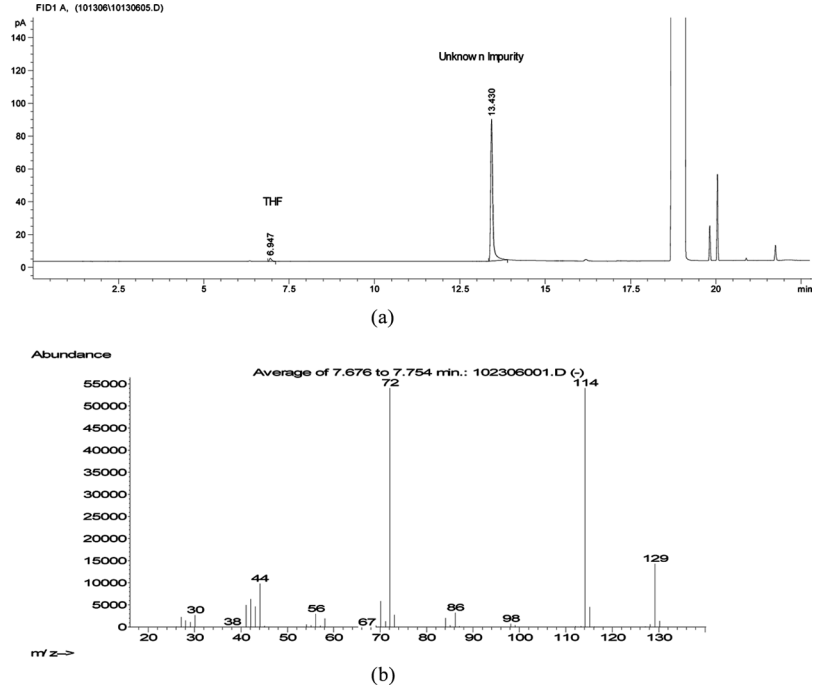
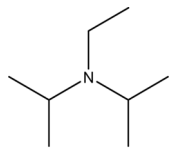



***Figure 7.*** Case Study C: (a) GC/FID chromatogram showing the unknown impurity. (b) The EI-MS spectrum of the impurity obtained from GC/MS experiments.

fragmentation of the molecular ions, whereas electrospray ionization is known to be a "soft" ionization technique.[13,17,18] It is relatively easy to compare EI-MS sample data to the library data set and search, mainly due to the following two facts. First, the EI MS data are acquired and cataloged into commercially available libraries at the standardized 70 eV, thus the mass spectra are relatively consistent regardless of differences in manufacturers and instrumentations. Secondly, since it is a hard ionization technique EI-MS yields fragmentation spectra characteristic of the compound.

The mass spectrum acquired for the unknown impurity is shown in Figure 7(b). The library search of the mass spectrum returned diisopropyl ethyl amine (DIEA, also known as Hunig's base) with the highest score. The structure is shown below. The MS spectrum was generated by EI, therefore, the molecular ion observed was at m/z 129, the molecular weight of DIEA. The ion at m/z 114 showed a loss of a methyl group, and the ion at m/z 72 was the result of methyl and isopropyl group loss from the molecular ion. A sample of Hunig's base was analyzed as a standard and the retention time and mass spectral data confirmed the identity of the impurity. Subsequently, appropriate washes were implemented to remove Hunig's base for the preparation of this intermediate.




$C_8H_{19}N$
Exact Mass: 129.15
Mol. Wt.: 129.24

Structure of diisopropyl ethyl amine (DIEA).

## CONCLUSION

Three case studies of impurity identification and control during the drug substance development were presented. It involved use of several analytical techniques complementing each other, including HPLC/UV, HPLC/MS, tandem mass spectrometry, preparative LC, HRMS, NMR, GC/FID and GC/MS.

Impurity identification of the case study A required a multidisciplinary approach, namely, generation of molecular ion and daughter ions using a range of MS/MS conditions, followed by preparative LC and reference synthesis by synthetic chemists, and

confirmation by HRMS and NMR. Subsequently, analytical methods were developed to follow the impurity throughout the synthetic pathway. In the case study B, the impurity proposed based on the tandem mass spectrometric data was immediately valuable and helped to improve process parameters. The availability of EI MS library for GC/MS made the impurity identification straightforward in the case study C.

It was demonstrated that by identifying the structure of the impurity, the origin of the impurity was pinpointed making elimination/reduction of the impurity achievable. Understanding of the impurity formation expands the knowledge of the process chemistry and enables control of the impurities.



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