Application Note

Pharma & Biopharma

How to Catch a Potential Mutagenic Impurity

Using an Agilent LC/MSD XT and Agilent InfinityLab Poroshell 120 HILIC-Z column for sensitive and reliable detection of dalfampridine impurities

Author

Kyle Covert
Agilent Technologies, Inc.

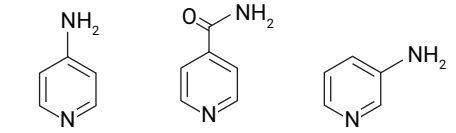
Abstract

This Application Note demonstrates the enhanced analytical sensitivity of using a mass selective detector (MSD) over UV detection alone, and the excellent separation and reliability of the Agilent InfinityLab Poroshell 120 HILIC-Z column, with compliance features provided by OpenLab CDS software. The United States Food and Drug Administration (FDA) has set the guidance threshold of toxicological concern (TTC) for potential mutagenic impurities to 1.5 µg/day. An Agilent 1260 Infinity II Prime LC coupled to an Agilent LC/MSD XT mass selective detector was used to detect two potential mutagenic impurities (PMIs) of a daily dose (20 mg) of dalfampridine with an instrument detection limit (IDL) of less than 16 pg for both compounds. This is nearly 100 times more sensitive than UV detection alone, and ensures that impurities can be detected for even small doses of pharmaceutical drugs. An InfinityLab Poroshell 120 HILIC-Z column was able to easily separate the compounds in less than five minutes. Injections over multiple days showed a %RSD of less than 7% for peak area, and less than 1% for retention times.

Introduction

Dalfampridine (4-aminopyridine (4AP)) is a novel drug used to treat patients with multiple sclerosis or other diseases affecting motor function. It has helped improve walking ability in adults by inhibiting augmented potassium channels. These channels release too much potassium, causing a decrease in action potential duration and amplitude.

Dalfampridine may contain impurities from the manufacturing process of the active pharmaceutical ingredient (API), which are potential mutagenic impurities. Specifically, two PMIs were targeted in this work: isonicotinamide and an isomer of the API itself, 3-aminopyridine, whose structures are shown in Figure 1. With a TTC for PMIs at 1.5 µg/day, a standard daily dose of 20 mg dalfampridine corresponds to no more than 75 ppm of PMIs in relation to the API[1,2]. Therefore, detection of these PMIs requires a sensitive, precise, and high-throughput methodology and instrumentation to meet the requirements.



4-Aminopyridine Isonicotinamide 3-Aminopyridine

Figure 1. API and two potential mutagenic impurities.

The API and PMIs are hydrophilic, and do not retain well on a typical reversed-phase liquid chromatography column. To obtain sufficient chromatographic separation, reversed-phase ion pairing can be used. However, this method requires reagents that are hard to remove from the system, and can cause ion suppression in the mass spectrometer, and this requires a dedicated LC. Therefore, a hydrophilic interaction liquid chromatography (HILIC) method was employed.

The InfinityLab Poroshell 120 HILIC-Z (zwitterionic) column offers the perfect solution for hydrophilic compound separation and detection. It is designed to retain polar compounds with superior robustness. The typical mobile phases are acetonitrile (ACN) and water with appropriate additives.

The Agilent single quadrupole LC/MSD XT can easily be incorporated into an HPLC stack, and is the perfect choice for impurity detection with mass confirmation alongside UV detection. It is specifically designed to be integrated seamlessly into the Agilent 1260 Infinity II LC or 1290 Infinity II LC system.

Experimental

Standards and chemicals

All reagents and solvents were HPLC or LC/MS grade. ACN was purchased from Honeywell (Morristown, NJ, USA). 4-Aminopyridine, isonicotinamide, 3-aminopyridine, ammonium formate, and formic acid were all purchased from Millipore-Sigma (Merck, Darmstadt, Germany). Ultrapure water was produced using a Milli-Q Integral system equipped with a LC-Pak Polisher and a 0.22 µm point-of-use membrane filter cartridge (EMD Millipore, Billerica, MA, USA).

Instrumentation

The LC/MSD XT system consists of the following modules:

- Agilent 1260 Infinity II Flexible Pump (G7104C)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Diode Array Detector HS (DAD) (G7117C)
- Agilent LC/MSD XT system (G6135CA)

The high-sensitivity diode array detector was used with a 60 mm Max-Light Cartridge Cell to use the maximum sensitivity of the UV detector in combination with the LC/MSD XT.

Sample preparation

API samples were prepared by dissolving 20 mg of 4AP into 1 mL of ACN in a 2 mL screw top sample vial (20,000 µg/mL). Stock solutions of the impurity standards were prepared by dissolving 10 mg of each into 100 mL of ACN in a volumetric flask (100 µg/mL). A series of serial dilutions was performed to make up 10, 1.0, 0.1, and 0.01 µg/mL solutions of the impurity standards. Separate samples of 20,000 µg/mL API standard were spiked with 150 µL of the previous impurity standards, respectively, to make ~1,150, 115, 11.5, and 1.15 ng/mL of impurity spiked API samples. The relation of impurities to the API in the samples was 75 to 0.075 ppm. This concentration range was chosen to cover the respective detection limits of the UV detector and LC/MSD XT in scan and selected ion monitoring (SIM) modes.

OpenLab CDS

Agilent OpenLab CDS software was used for data acquisition, processing, and reporting. OpenLab CDS provides compliance features that support data integrity with US FDA 21 CFR Part 11, EU Annex 11, and other similar regulations. The 1260 Infinity II LC and LC/MSD XT are designed to ensure reliable and robust LC/MS for routine applications.

Results and discussion

The integrated divert valve ensures system robustness

MS spectral information facilitates the detection and confirmation of impurities. However, repeated sample injection of high API concentrations may slowly cause contamination of the MS ionization source over time. This challenge can be minimized using the built-in diverter valve included in Agilent mass spectrometers. The valve may be set to divert the column eluent flow to waste during the elution of the highly concentrated main peak (20,000 µg/mL). During LC method development, we ensured that the main API peaks were well separated from the rest of the impurities. The diverter valve switching is smooth, and maintains retention time reproducibility.

Table 1. 1260 Infinity II LC method parameters.

Parameter	HPLC Set Value
Column	InfinityLab Poroshell 120 HILIC-Z, 2.1 × 150 mm, 2.7 µm, PEEK lined at 35 °C
Mobile Phase A	10 mM ammonium formate + 0.02 % formic acid (pH 5.0)
Mobile Phase B	0.1% formic acid in acetonitrile (ACN)
Gradient	Time %B 0 95 2 90 4 60 5 60
Post Run	5 minutes
Flow Rate	0.5 mL/min
Injection Volume	1 µL
Detection UV	(265, 10/ref. 360, 80) nm

Table 2. Agilent LC/MSD XT parameters.

Parameter	Single Quadrupole Set Value
Ion Source	ESI+
Peak Filter	0.02 minutes
Scan/Dwell Time	Scan 250 ms, SIM 50 ms
Drying Gas Temperature	350 °C
Gas Flow	10 L/min
Nebulizer Pressure	40 psi
Capillary Voltage	3 kV
Fragmentor Voltage	135 V
Scan Range	*m/z* 80 to 300
SIM Ions	*m/z* 95.2, 123.1
Divert to Waste	3.5 to 5 minutes

Table 3. List of compounds of interest with their *m/z*, retention time, and log P values.

Compound	*m/z*	Retention Time	log P
API (4-Aminopyridine)	95.2	3.98 (onset)	0.26
Impurity I (Isonicotinamide)	123.1	1.14	-0.28
Impurity II (3-Aminopyridine)	95.2	1.96	-0.02
Nicotinamide	123.1	1.52	-0.11

A 1 µL injection of 20,000 µg/mL API standard without impurities was performed with only UV detection. Figure 2 shows the UV profile of the API to determine the time window to divert the LC flow to waste, and protect the MS detector. This same sample is injected again, shown in blue in Figure 3, diverting the API to waste from 3.5 to 5.0 minutes. This was performed to see if there was any detectable abundance of the target impurities in the API standard alone.

There are two peaks detected in the pure API at 1.523 and 3.146 minutes. The first peak has an *m/z* of 123.1, and is suspected to be nicotinamide, an isomer of the precursor (isonicotinamide), commonly used in the reaction to synthesize dalfampridine. This is further confirmed by the log P values, a measure of a compound's hydrophobicity, which places nicotinamide between impurity I and impurity II. The second peak detected in the API standard has an *m/z* of 95.2, which gives it the same mass as the API and impurity II. However, the retention time for this peak does not match with the API or impurity II, so it is possibly another isomer of the API, 2-aminopyridine.

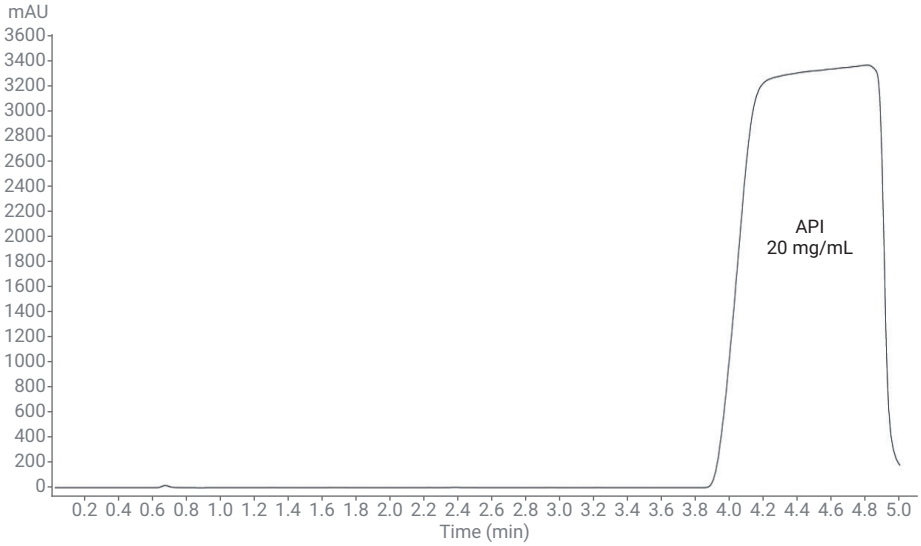


Figure 2. UV chromatogram of the API (20 mg/mL).

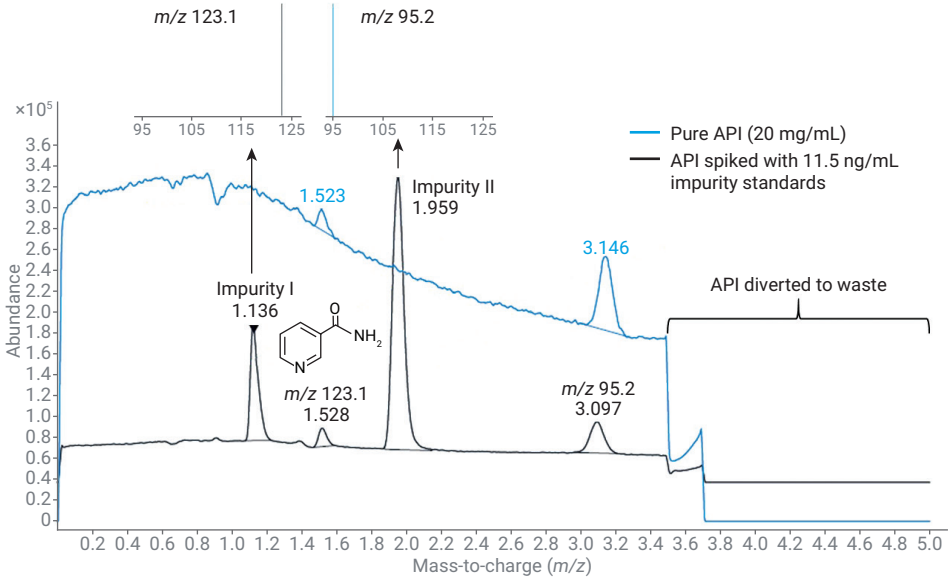


Figure 3. Mass chromatogram (SIM TIC), from mass detector, of the pure API (20 mg/mL) shown in blue, and the same API spiked with 11.5 ng/mL of impurities, relative to the API, shown in black for comparison. MS spectra of the impurity peaks are inlayed. The peak at 1.528 has been identified as nicotinamide, present in the API standard.

Enhanced analytical sensitivity of LC/MSD XT

A 1 µL injection containing 11.5 ng/mL of impurities shows no discernable peaks for the impurities in the UV chromatogram shown in Figure 4A. Impurities were only detected in the UV at concentrations above 20 ng/mL. An inset in Figure 4A shows the UV chromatogram of a 1 µL injection at a concentration of 115 ng/mL of impurities. Impurity II and the API are well separated by the InfinityLab Poroshell 120 HILIC-Z column.

Figure 4B features the SIM TIC, and the impurities are easily distinguished from the noise as peaks at 1.136 and 1.950 minutes. SIM mode only allows single *m/z* ions to pass in the quadrupole. Therefore, the quadrupole spends significantly more time analyzing select ions, enabling greatly enhanced analytical sensitivity versus scanning across an *m/z* range.

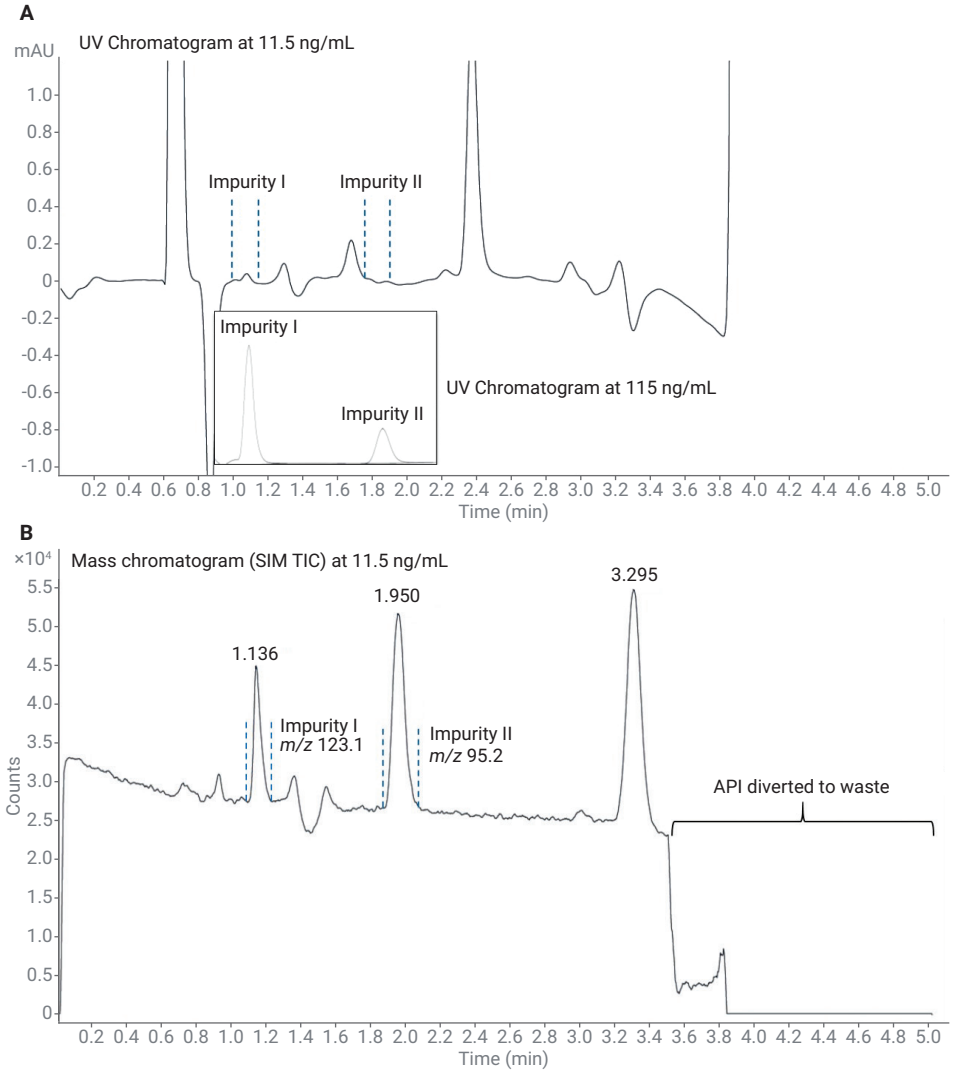


Figure 4. Two chromatograms of the API spiked with 11.5 ng/mL of impurities: A) Zoomed-in region of the UV chromatogram with the expected retention time of the impurities highlighted with dashed lines. The inset is a UV chromatogram from a higher concentration of impurities, confirming retention times. B) Mass chromatogram (SIM TIC), *m/z* 123.1 and 95.2, impurity I and II, respectively, at 11.5 ng/mL.

LC/MSD XT with improved analytical sensitivity and higher precision for low-level impurities

Because the masses of impurity II and the API are the same, standards for these compounds were analyzed individually to obtain their specific retention times. The retention times of the impurities were determined by a 1 µL injection of 150 ng/mL of the standard impurity, as shown in Figure 5. An injection containing 11.5 ng/mL of impurities is also shown to demonstrate that the spiked samples have the same retention times.

The API was analyzed with a series of 1 µL injections where the impurities were spiked into the API at 115, 11.5, and 1.15 ng/mL concentrations, and are shown in Figure 6. At the 115 ng/mL level, the impurities can be determined by UV at approximately the limit of quantitation, and are easily distinguished by the LC/MSD XT in scan mode. At the 11.5 ng/mL level, the impurities cannot be seen in the UV chromatogram, and are not distinguishable in the scan mode of the mass spectrometer. However, the LC/MSD XT is easily able to detect the impurities in SIM mode.

The lowest concentration analyzed was 1.15 ng/mL for both impurities. The signal-to-noise ratio (S/N) was 12 and 28 for impurities I and II, respectively, using SIM mode. This demonstrates that the LC/MSD XT in SIM mode has a detection limit 100x greater than UV detection for these compounds.

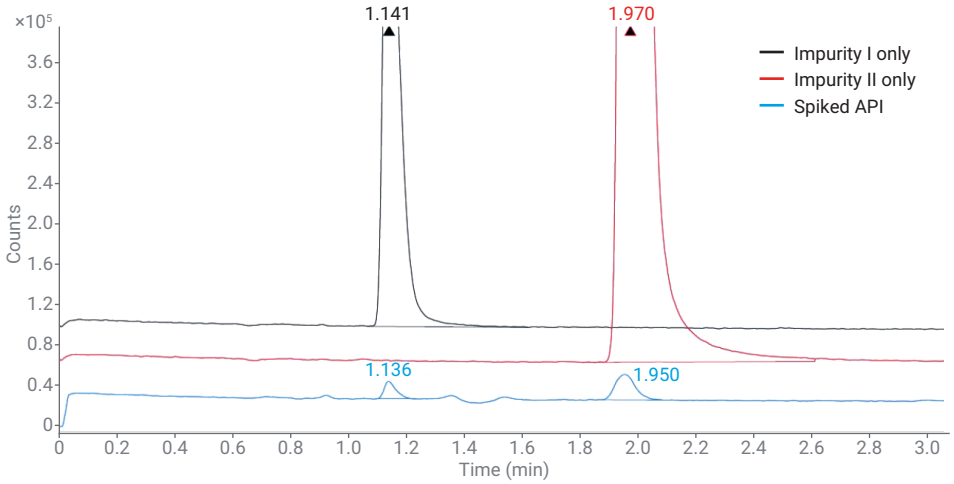


Figure 5. Stacked mass chromatogram (SIM TIC) of the individual impurities and a 11.5 ng/mL spiked sample. The pure impurity RTs were compared to the spiked sample to ensure that the RT remains consistent in the presence of a large amount of API.

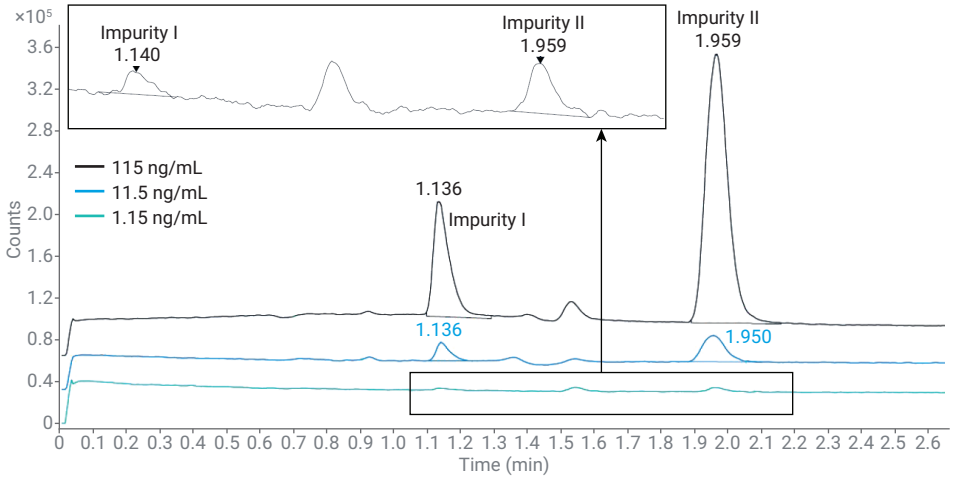


Figure 6. Stacked mass chromatogram (SIM TIC) of the impurities spiked into the API at 115, 11.5, and 1.15 ng/mL concentrations. The inlay is a zoomed portion of the SIM chromatogram at 1.15 ng/mL (1.15 pg on-column) to show that the impurities are still detectable at this concentration.

InfinityLab Poroshell 120 HILIC-Z and LC/MSD XT detection sensitivity (IDL <16 pg)

A good measure of the overall analytical sensitivity of a method can be established by calculating the instrument detection limit (IDL)[3,4]. The IDL not only takes the detector of choice into account, but also the entire HPLC system from pump to column by comparing peak RSDs over multiple injections[3,4]. The IDL was determined for the LC/MSD XT operating in SIM mode for the impurity peaks. The IDL was determined to be 15.40 pg for impurity I and 5.65 pg for impurity II from 10 replicate 5 µL injections of a 20 ng/mL sample containing only the impurities, as shown in Figure 7.

The reproducibility of the 1260 Infinity II Prime LC coupled to the LC/MSD XT with the InfinityLab Poroshell 120 HILIC-Z column shows excellent performance, as shown in Figure 8, by performing three injections of the same sample over three days, approximately 24 hours apart. The %RSD of the peak areas were 6.7% for impurity I and 4.1% impurity II. Both impurities have a %RSD of retention times of less than 1%.

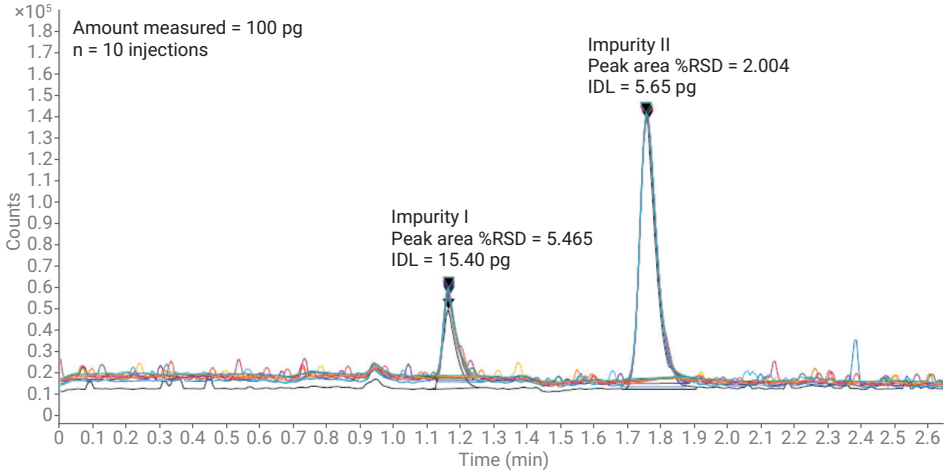


Figure 7. SIM chromatograms of 100 pg injections containing only the impurities. Ten injections were performed to calculate the IDL for the individual impurities. The IDL was determined to be 15.40 pg for impurity I and 5.65 pg for impurity II.

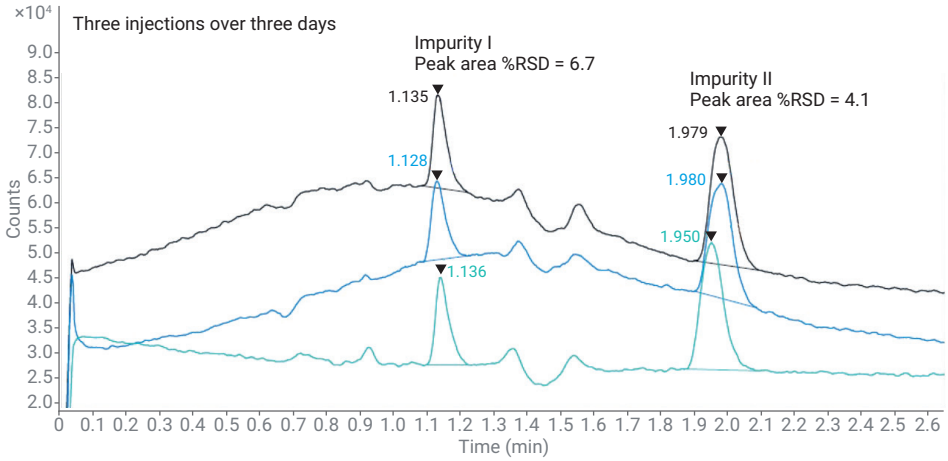


Figure 8. Stacked mass chromatogram (SIM TIC) of a 11.5 ng/mL spiked API sample injected over the course of three days. %RSDs of the peak areas are shown to display the reproducibility of the LC/MSD XT system with the Agilent InfinityLab Poroshell 120 HILIC-Z column.

Conclusion

Identifying potential mutagenic impurities requires a sensitive and reliable instrument with the capability of detecting high concentrations of API. The InfinityLab series of consumables, LC modules, and the LC/MSD XT offers a reliable, sensitive, and robust system that can easily be incorporated into an impurity analysis workflow, in a compliant environment using OpenLab CDS. UV detection alone is not sensitive enough to detect the low levels of PMIs, underlining the need for an MSD, as demonstrated in this study. Together with the 1260 Infinity II Prime LC System, the LC/MSD XT obtained instrument detection limits of 15 pg for impurity I, and 6 pg for impurity II. The InfinityLab Poroshell 120 HILIC-Z column is perfectly suited for separation of polar compounds, and it has been shown to produce consistent and reliable separations.

References

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