Application Note

Pharmaceutical Small Molecules

Agilent

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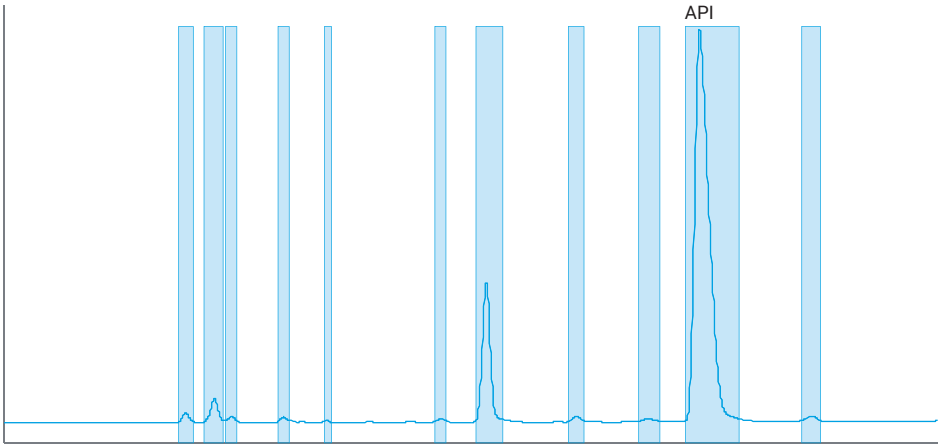
Isolate and Reanalyze Pharma Impurities with the Agilent 1290 Infinity II Autoscale Preparative LC/MSD System



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Abstract

Isolation of impurities for further characterization is a routine task in the pharmaceutical industry, and is frequently carried out using preparative high-performance liquid chromatography (HPLC). This application note presents a small-molecule purification workflow including method development on an analytical scale, upscaling to preparative conditions, fraction collection, and fraction reanalysis. All steps are carried out on a single system, the Agilent 1290 Infinity II Autoscale Preparative LC/MSD System. Combined with the features of Agilent OpenLab CDS ChemStation, this system minimizes manual steps of the workflow, reducing labor and error sources.

Introduction

Organic impurities are often encountered during synthesis or long-term storage of small molecule pharmaceuticals. Many common degradation products and intermediates from the synthesis of registered pharmaceuticals are described in the different pharmacopoeias. Whenever new molecules are developed or unknown impurities are detected, these compounds need to be isolated and concentrated for further characterization. Separation of impurities from a main compound is frequently done by HPLC. For isolation of large amounts of unknown substances, preparative-scale HPLC is the method of choice.

A preparative purification workflow usually starts with method development in analytical scale. Once suitable stationary and mobile phases are found and the separation gradient has been optimized, conditions are scaled up to preparative scale and fractions are collected. Every compound isolated by the fraction collector then needs to be homogenized and reanalyzed. Some steps of this workflow require manual interaction (e.g., moving samples from the analytical to the preparative system or preparing fractions for re-injection on an analytical system).

The 1290 Infinity II Autoscale Preparative LC/MSD System combines analytical and preparative hardware in a single system. Fractions collected by the combined autosampler/fraction collector, for example, can be homogenized and re-injected without being moved from the fraction bed. This application note demonstrates how the smart interplay of hardware and software can save time and labor during a typical purification workflow. Eliminating manual steps also helps remove error sources from the process.

Experimental

Instrumentation

For this experiment, an Agilent 1290 Infinity II Autoscale Preparative LC/MSD System comprising the following modules was used:

- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with 50 mL pump heads (option #202)
- Agilent 1260 Infinity II Quaternary Pump (G7111B)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B) with 5 mL preparative sample loop (option #241)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with 3 mm preparative quartz flow cell (option #082)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm standard cell (option #018)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A) with knitted delay coils for 21.2 mm id columns/15 to 40 mL/min flow (option #210)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-Position/14-Port preparative-scale valve head (G4738A)
- Agilent InfinityLab LC/MSD XT (G6135B)

Columns

- **Analytical column:** Agilent Prep-C18 Scalar, 4.6 × 100 mm, 5 µm (part number 449905-902)
- **Preparative column:** Agilent Prep-C18, 21.2 × 100 mm, 5 µm (part number 449905-702)

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, version C.01.10 [272]

Solvents and sample

All solvents used were LC grade and purchased from VWR (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Ibuprofen sodium salt was purchased from Sigma (Taufkirchen, Germany) and deliberately deteriorated. A 50 mg/mL solution was prepared in acetonitrile/water (15/85, v/v) and diluted 1:10 for analytical injections.

Method settings

The separation gradient was developed for analytical scale first and then transferred to preparative conditions using the HPLC calculator developed at University of Geneva.[1] Tables 1 and 2 present detailed HPLC and MSD method settings, respectively.

Table 1. Chromatographic conditions of analytical and preparative runs.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	1.5 mL/min	31.86 mL/min
Gradient	0.00 min to 15% B 8.00 min to 95% B 8.20 min to 15% B	0.00 min to 15% B 0.75 min to 15% B 8.75 min to 95% B 9.00 min to 15% B
Stop Time	9.5 min	10 min
Post Time	1.3 min	1.3 min
Injection Volume	10 µL (scouting) 50 µL (fraction reanalysis)	3,000 µL
Sampler Method Preset	Plug setting 2 180 µL postsample plug 25% methanol in water	Plug setting 2 180 µL postsample plug 25% methanol in water
Temperature	Ambient	Ambient
UV Detection	254 nm 5 Hz data rate	254 nm 5 Hz data rate
MS Detection	Signal 1: Positive SIM, 56.0% cycle time *m/z* 165.1, 177.1, 179.1, 193.1, 206.1, 207.1, 221.1, 223.1 Signal 2: Negative SIM, 44% cycle time *m/z* 163.1, 175.1, 177.1, 191.1, 205.1, 221.1	Signal 1: Positive SIM, 56.0% cycle time *m/z* 165.1, 177.1, 206.1, 207.1 Signal 2: Negative SIM, 44% cycle time *m/z* 163.1, 175.1, 205.1
Split Ratio to MSD	Full flow	1,500:1 (mode M1) 1.00 min ON 9.00 min OFF
Fraction collection	Not applicable	Peak-based: 3.00 to 9.00 min Threshold 5 mAU Up-/downslope 5 mAU/s

Table 2. MSD spray chamber settings.

Parameter	Value
Make-Up Solvent	0.1% formic acid in methanol/water (70/30)
Make-Up Flow	1.5 mL/min
Ionization Source	Agilent Jet Stream Electrospray
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Drying Gas Flow	11.0 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11.0 L/min
Capillary Voltage	±4,000 V
Nozzle Voltage	±600 V

Results and discussion

A deteriorated ibuprofen sample was analyzed by HPLC using a linear gradient in analytical scale. The chromatogram revealed that the sample was degraded (Figure 1). To investigate the impurities, all unknown peaks should be collected by means of preparative-scale liquid chromatography to provide enough material for further analyses. Conditions of the analytical run were scaled up using a HPLC gradient transfer calculator[1] in order to preserve retention times and resolution of the analytical chromatogram. Injection volume under preparative conditions was adjusted to 15 mg sample on-column first to confirm the scale-up.

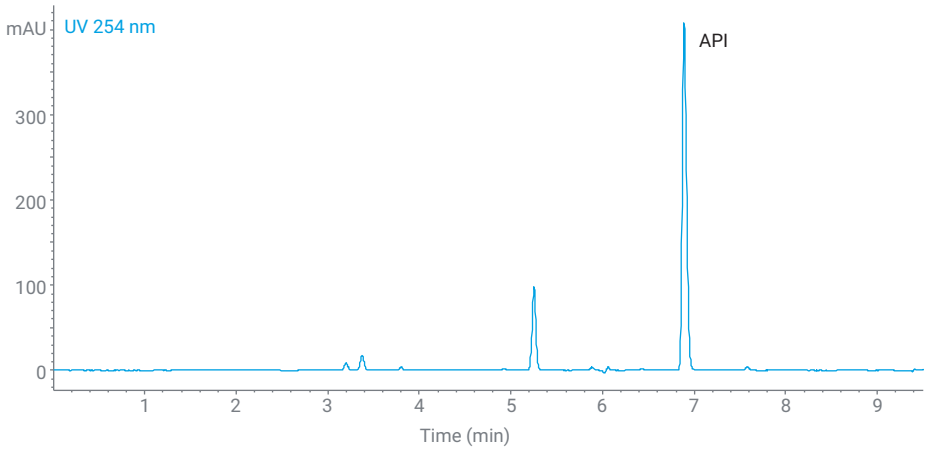

Figure 1. Separation of the sample in analytical scale. Multiple peaks indicate that the active pharmaceutical ingredient (API) has deteriorated.

Figure 2 shows an overlay of the analytical and preparative separation. The peak elution pattern in both chromatograms shows that the scale-up was successful. For fraction collection, injection volume was further increased tenfold to 150 mg sample on-column. Collection was triggered on the UV signal with a combination of threshold and slope. Ten peaks were successfully collected (see Figure 3).

After successful collection, fractions were reanalyzed under analytical conditions with mass-selective detection. The Purify view in ChemStation data analysis features a tool to collect fractions for re-injection and automatically set up a re-injection sequence (see Figure 4). Fractions that shall be reanalyzed can be selected and added to the sequence by a single click. Sample name and information will be filled in automatically with reference to the preparative purification run. A default method for re-injection can be preselected. Once the re-injection sequence is generated and saved, it can be loaded into an online ChemStation. All sequence parameters remain editable until submission, allowing, for instance, for changes to the injection volume or addition of blank and reference runs to the sequence.

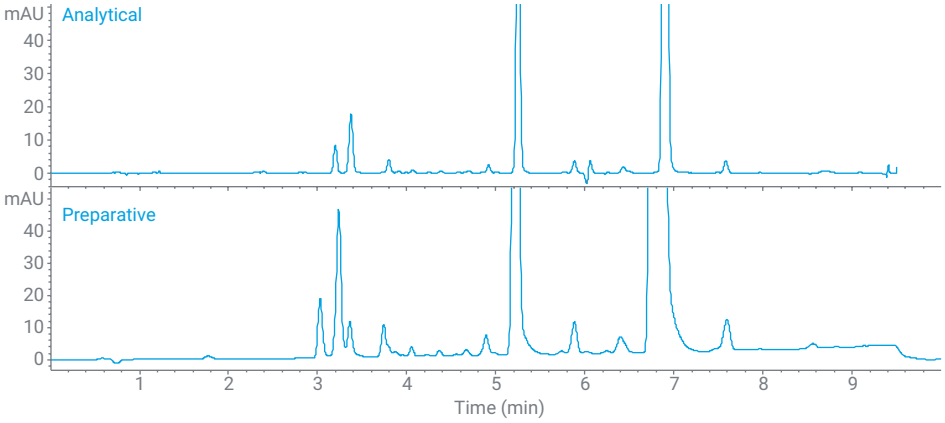

Figure 2. Chromatograms of analytical- and preparative-scale separation of the sample.

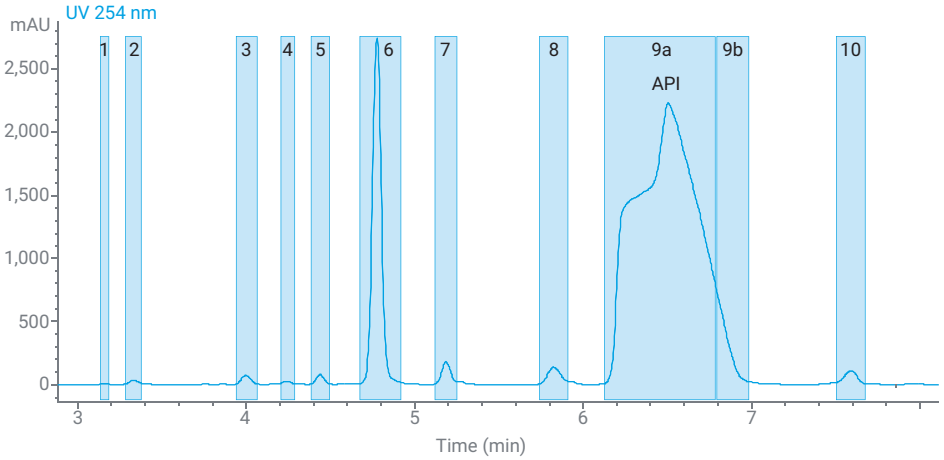

Figure 3. Chromatogram cut-out of the preparative-scale separation with fraction collection. Blue bars represent fraction collection events. Despite heavy overloading, the API was successfully separated and collected.

Using the 1290 Infinity II Preparative Open-Bed Sampler/Collector enabled setting up and running the re-injection sequence without any manual handling of the fractions. The method that was chosen for reanalysis contained a homogenization step prior to injection, which was carried out automatically by the autosampler. The importance of fraction homogenization is described in detail in another technical overview.[2]

Purity of each reanalyzed fraction was determined by the ratio of target peak area versus total peak area at 254 nm after blank subtraction. Most fractions were very pure (>99% target peak area). Fractions 2, 3, and 8, however, had a purity of only 45 to 70% (see Figure 5). These impurities had not been detected during analytical scouting. After scale-up to preparative conditions, resolution or selectivity of the column was not high enough to separate these compounds. Looking at the mass spectra of the impurities, the most abundant ions indicate that the detected compounds might be ibuprofen aldehyde, and two degradation products of ibuprofen or its impurities listed in pharmacopoeias. For want of certified reference standards, further elucidation of these impurities was not pursued. Importantly enough, software-supported fraction reanalysis revealed that not all fractions were as pure as the preparative chromatogram suggested. These results confirm that fraction reanalysis should be a routine part of any purification workflow. With the 1290 Infinity II Autoscale Preparative LC/MSD System used for these experiments, this step can be executed with minimum effort and even without touching the collected fractions.

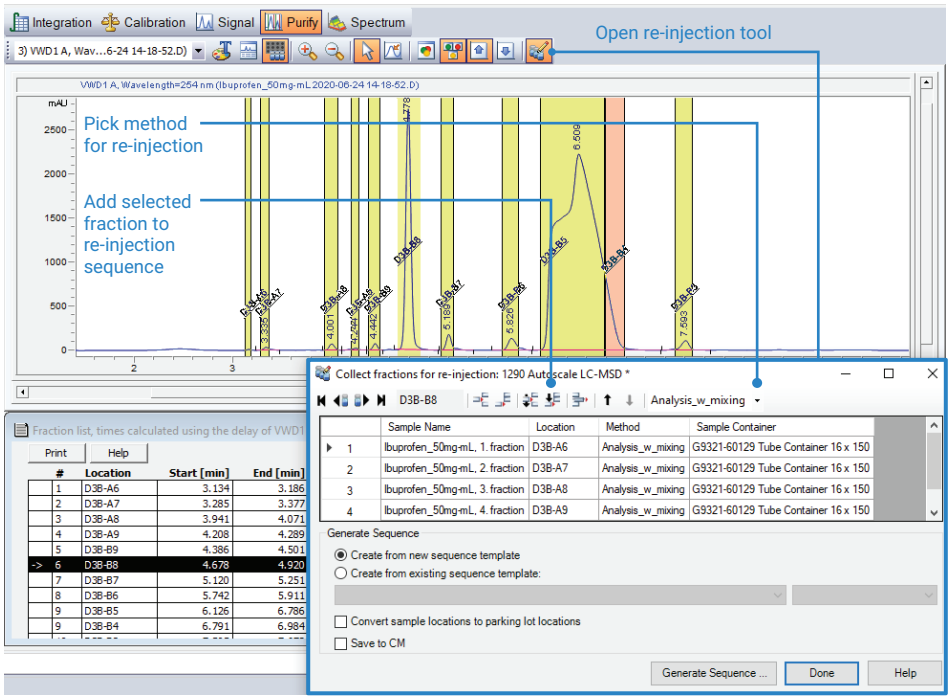

Figure 4. Re-injection tool in Agilent ChemStation data analysis. Single fractions can be hand-picked and added to a re-injection sequence. Sequence parameters are prefilled but remain editable.

Conclusion

Using a 1290 Infinity II Autoscale Preparative LC/MSD System, a pharmaceutical sample was separated in analytical scale. The optimized method was scaled up to preparative conditions, maintaining separation of all peaks. Preparative purification of all sample components was carried out using the preparative flow path of the system. All fractions were subjected to homogenization and reanalysis. Switching back to the analytical flow path and using software support, these steps could be executed with minimum user interaction, requiring no manual handling of the fractions. Results show that three fractions were not as pure as suggested by the preparative chromatogram, demonstrating that fraction reanalysis is crucial for a confident purification. With the system presented here, the entire workflow can be executed faster and with less user interaction.

References

1. Guillarme, D. *et al. Eur. J. Pharm. Biopharm.* **2008**, *68*, 430–440.
2. Spuling, I.; Rieck, F. Spotting Fraction Impurities with More Confidence Using the Agilent 1290 Infinity II Preparative Open-Bed Sampler/ Collector. *Agilent Technologies technical overview*, publication number 5994-1643EN, **2019**.

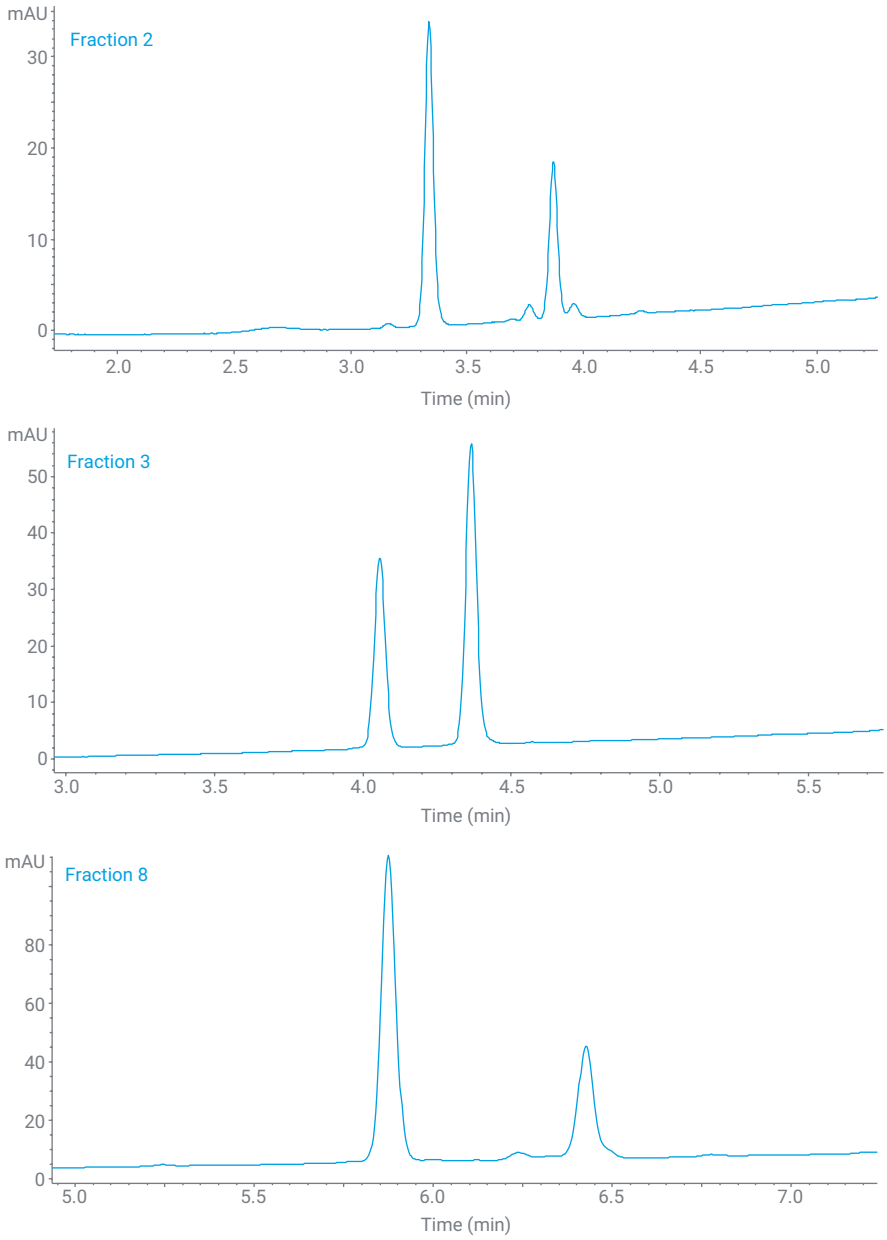

Figure 5. Chromatograms of fraction reanalysis. Some impurities were only discovered by reanalyzing the collected fractions with LC/MS.

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