A New Workflow for Purifying HPLC Samples Starting with Scouting Gradient Methods

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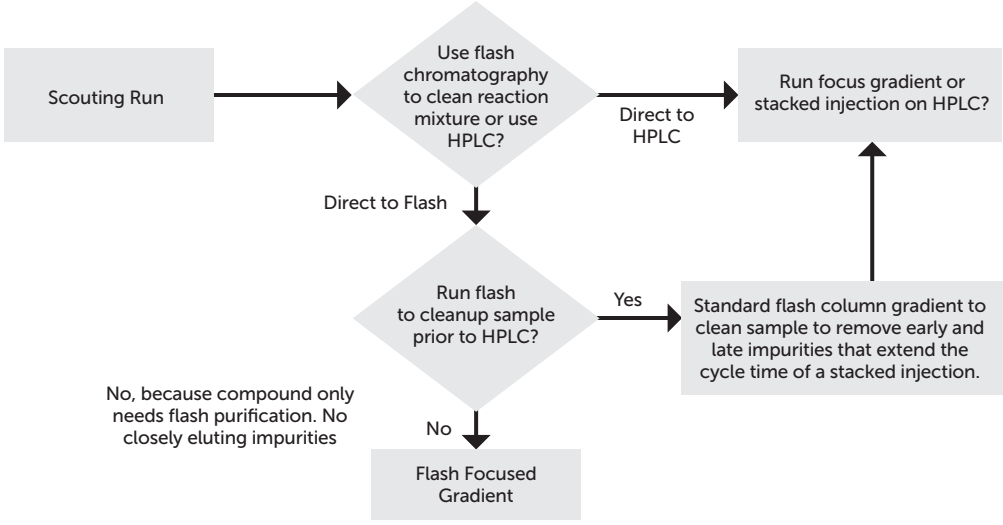
Abstract

A workflow is suggested for preparative high performance liquid chromatography (HPLC) to purify synthesized compounds. In addition to verifying that the reaction ran as expected, scouting gradients using an analytical HPLC system are used to evaluate the resolution of compounds. The resolution is used to determine whether HPLC is required to purify the sample or whether flash chromatography is sufficient. Different columns and solvent systems are often tested with scouting gradients to find the combination giving the best resolution. Evaluating the peak shape may indicate that the sample needs purification with a buffer. The scouting gradient retention time is used to calculate either a focused gradient or an isocratic method that can be used for stacked injections. The scouting gradient data and the amount of sample to be purified can be used to suggest whether the samples need pre-purification processes (such as flash chromatography or a general gradient method) prior to a focused gradient or a stacked injection.

Background

Purification is needed to purify desired compounds from impurities such as side-reactions, unreacted starting compounds, and other impurities. One of the most common purification methods uses HPLC. Method development in HPLC generally begins with a scouting gradient that is used to determine if the reaction proceeded as expected and the extent of impurities in the reaction mixture. A scouting gradient can also be used to determine a purification method[1] and whether the purification method is likely to have good peak shape. In addition to focused gradients, isocratic methods for stacked injections can be determined. The workflow requires no extra steps and reduces the amount of time and solvent needed compared to estimating a gradient method.



Scouting Run

Use flash chromatography to clean reaction mixture or use HPLC?

Direct to HPLC

Run focus gradient or stacked injection on HPLC?

Direct to Flash

Run flash to cleanup sample prior to HPLC?

Yes

Standard flash column gradient to clean sample to remove early and late impurities that extend the cycle time of a stacked injection.

No, because compound only needs flash purification. No closely eluting impurities

No

Flash Focused Gradient

Figure 1—Proposed workflow for preparative purification

Scouting runs and focused gradients

Scouting gradients are commonly run on reaction mixtures which provide the following information:

- Did a reaction occur?
- Extent of by-products (purity of reaction mixture).
- Compound identification (with mass spectrometer).
- Will the compound elute during preparative chromatography using a given column and solvent? (When matching columns used).

Scouting gradients can also provide additional information:

- An indication that a solvent modifier is needed. This is determined by peak shape. An asymmetric peak in a scouting run will have worse asymmetry when the gradient is changed to a focused or isocratic method.
- A focused or isocratic gradient method.
- Whether a compound needs preliminary cleaning prior to running a stacked injection.
- An appropriate injection solvent.

Method development

A scouting gradient can be used to generate either a focused gradient or an isocratic method by applying an apparent gradient delay determined by using the Time-on-Target algorithm.[1] The calibration is programmed into Teledyne LABS chromatography systems. The analytical scouting gradient calibration for one column type and solvent system will work with other columns so long as the column dimensions and the scouting gradient method are the same. The scouting gradients for all Teledyne LABS columns are programmed into PeakTrak®.

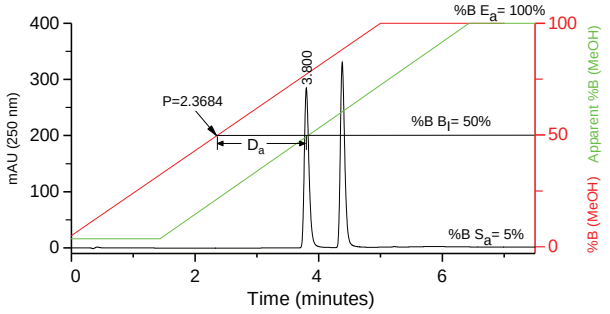
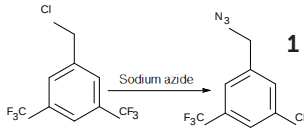


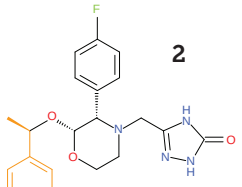
Figure 2—Analytical scouting run with apparent gradient delay measured. The second eluting peak would have a elution method of 60% B solvent.

Analytical to preparative method development

1-(Azidomethyl)-3,5-bis-(trifluoromethyl)benzene **(1)** is an intermediate to an NK1-II antagonist **(2)** and investigational compounds **(3)** and **(4)**.[1] It was synthesized according to a flow chemistry technique using Teledyne LABS ReaXus pumps.[2]



1



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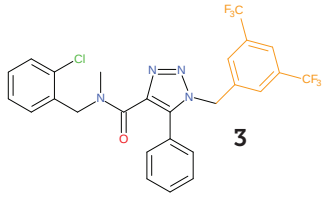

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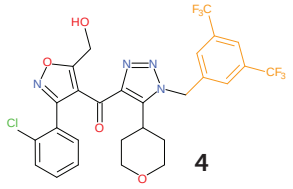

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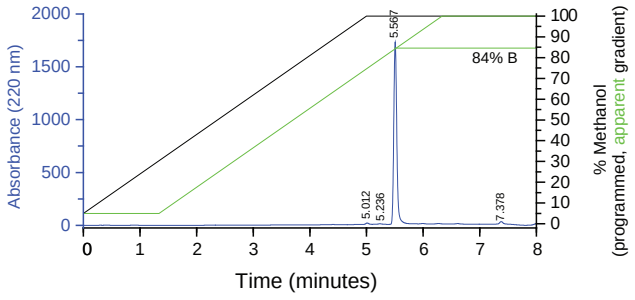
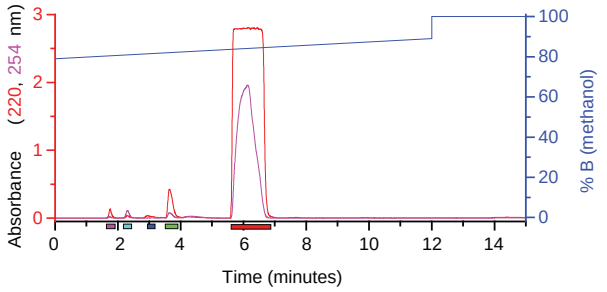
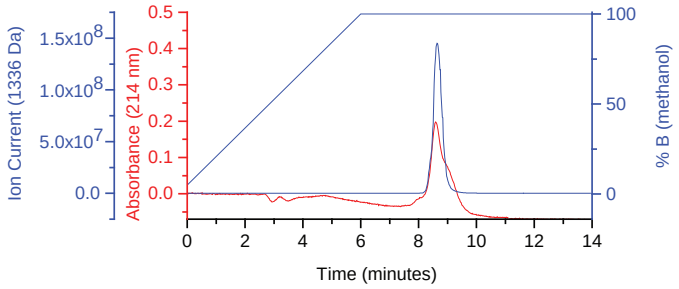


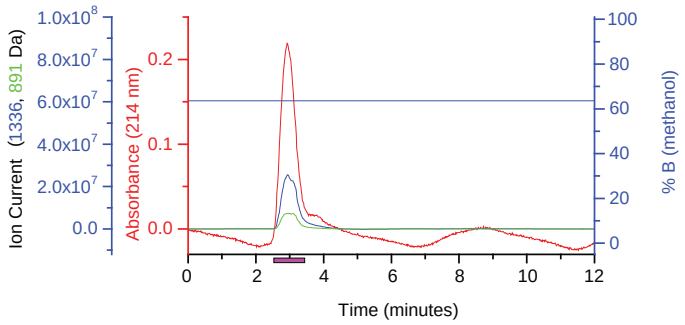
Analytical scouting gradient of synthesized 1-(Azidomethyl)-3,5-bis-(trifluoromethyl) benzene. Green line depicts the apparent gradient. A RediSep® Prep 2 x 50 mm C18 column was used.



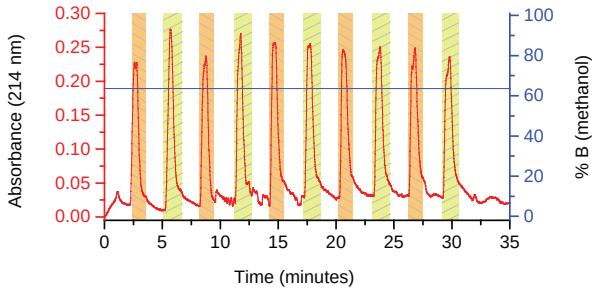
Preparative run of the compound run in the figure left using a gradient calculated on a Teledyne LABS HP150 HPLC system using the analytical scouting run in the chromatogram above. One gram of sample was loaded on a RediSep® Prep 20 x 150 mm column.



Scouting run shows impure peptide as shoulders around the peak detected with the PurIon™ mass spectrometer. An isocratic method was calculated from the scouting run using PeakTrak software and a 10 x 150 mm RediSep Prep C18 column. The calculated method used 63.6 % methanol in 50 mMol ammonium carbonate at pH 7.0.



Stacked injection used peak collection to determine the collection time windows. The peptide was collected only within the time windows. Only two injections (the scout and the isocratic run above) were needed to generate the stacked injection below. The calculated gradient suggested a mixture of methanol and water would be a good injection mixture. DMSO was avoided as the DMSO peak would increase the stacked injection cycle time.



Each colored band corresponds to a stacked injection. Stacking injections saved 65 minutes compared to performing multiple separate runs.

Conclusion

The procedure described herein follows the workflow used in most synthetic labs except the scouting gradient is used to determine an isocratic or focused purification method with no intermediate trial runs. An efficient focused gradient is calculated for flash or preparative chromatography from scouting runs on analytical HPLC or the preparative system itself. The scout run is sufficient to calculate an efficient focused gradient, and only one more run is needed to create stacked injections.

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[1] **Silver, J.** "Overview of Analytical-to-Preparative Liquid Chromatography Method Development." *ACS Combinatorial Science,* **2019**, *21* (9), 609–613. DOI: 10.1021/acscombsci.8b00187

[2] **Kopach, M.E., Murray, M.M, Braden, T.M., Kobierski, M.E., Williams, O.L.** "Improved Synthesis of 1-(Azidomethyl)-3,5-bis-(trifluoromethyl)benzene: Development of Batch and Microflow Azide Processes." *Org. Process Res. Dev.* **2009**, *13*, 152–160. DOI:10.1021/op800265e

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