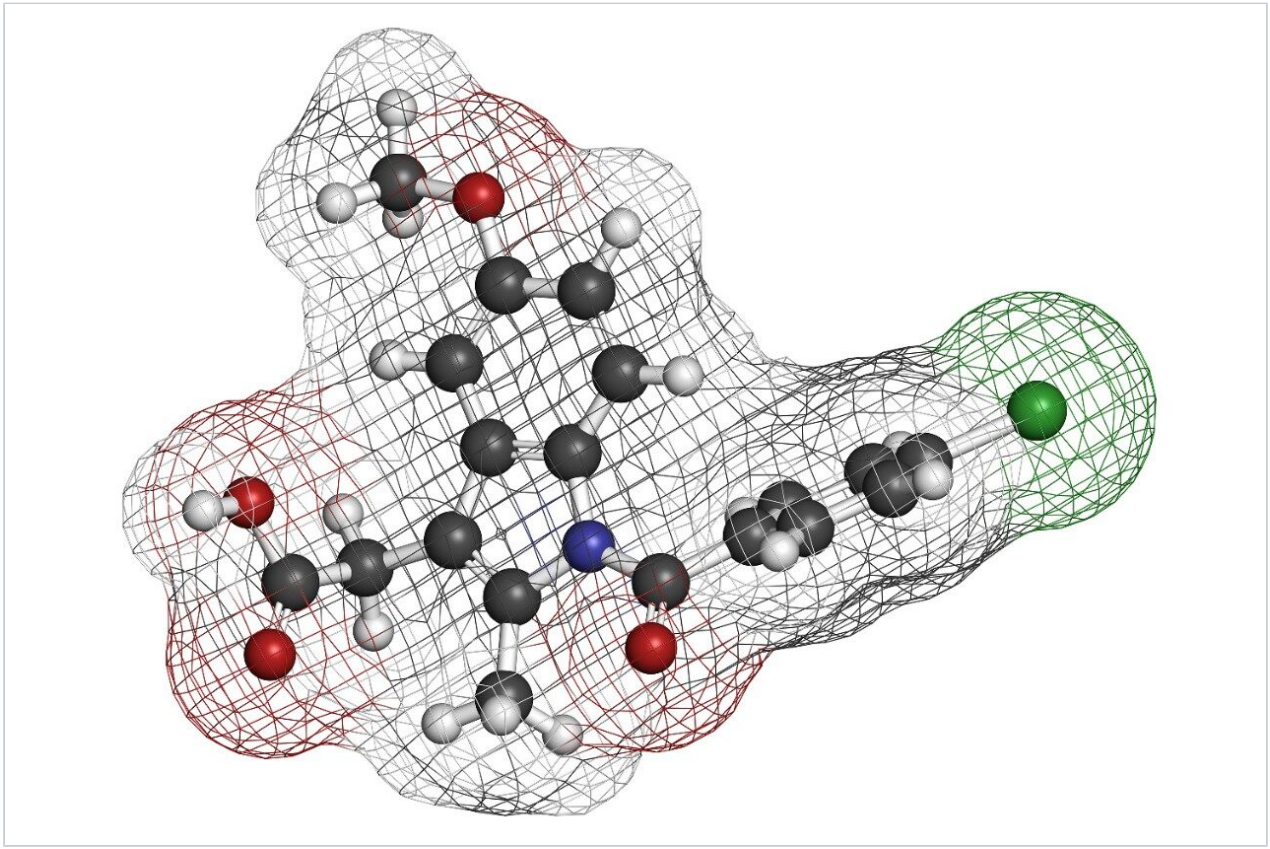
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Nota applicativa

Transfer of a UPLC Screening Method to Preparative HPLC Purification using CSH Column Technology and Focused Gradients

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Abstract

In this application note, we demonstrate a rapid UPLC screening protocol using high and low pH on a charged-surface hybrid (CSH) column and subsequent scale-up to an efficient preparative HPLC purification using focused gradients.

Benefits

- Rapid screening of synthetic reaction mixtures using UPLC and sub-2-µm particle columns
- Increased analytical confidence from the improved peak shape and retention time stability seen with pH switching using
  CSH Technology
- Direct transfer from UPLC screening to preparative HPLC, alleviating the need for time-consuming method redevelopment
- Faster purification throughput and cleaner samples using focused gradients

Introduction

Reaction screening and purification are essential steps in small molecule pharmaceutical discovery labs. Cleaner samples for subsequent synthesis can reduce reaction by-products and facilitate the critical final purification of material for pharmaceutical profiling and toxicology studies. Discovery chemistry facilities often have dedicated instruments for analytical scale screening of reactions to allow for quick monitoring of reaction progress. Lengthy screening methods on these instruments can cause excess analytical backlog and/or delays in judging when to stop a proceeding reaction. These delays can result in excess side-product formation and necessitate timeconsuming repeat synthesis, or require a more complex purification strategy. Next, the use of different column chemistries between analytical screening and larger-scale purification frequently results in redevelopment of the method at the preparative HPLC scale. Selectivity differences between the two column stationary phases may also necessitate target peak re-identification, resulting in a more complex purification strategy. Finally, development of a method to separate all the peaks in the mixture can be lengthy and inefficient, and is often unnecessary when targeting only a few peaks of interest for purification.

In this application note, we will demonstrate a rapid UPLC screening protocol using high and low pH on a charged-surface hybrid (CSH) column and subsequent scale-up to an efficient preparative HPLC purification using focused-gradients. The CSH column is chosen for its improved peak shape for basic compounds, wide pH range capability, and retention time stability with routine pH switching. The use of the same column stationary phase on the preparative scale maintains the separation selectivity from the UPLC screening run, alleviating the need for method redevelopment . After calculating the focused gradient from the UPLC screening run, the separation can be directly transferred to preparative HPLC for rapid purification of the compound of interest. The use of focused gradients in preparative HPLC allows for cleaner purification of the target compound in a shorter run time compared to the full gradient, ultimately resulting in higher sample purification throughput.

Experimental

ACQUITY UPLC Conditions

Mobile Phase A1:	0.1% formic acid in water
Mobile Phase A2:	0.1% formic acid in acetonitrile
Mobile Phase B1:	0.1% ammonium hydroxide in water
Mobile Phase B2:	0.1% ammonium hydroxide in acetonitrile
Column:	ACQUITY UPLC CSH C_{18}, 2.1 x 30 mm, 1.7 µm, part number 186005295
Run Time:	3 min
Column Temp.:	30 °C
Detection:	UV at 210 and 254 nm
Needle Wash:	95:5 acetonitrile:water

Sample Purge:	90:10 water:acetonitrile
Flow Rate:	0.9 mL/min
Injection Volume:	0.2 µL
MS Detection:	ToF-MS, ESI+ mode
Scan Range:	100-1000 amu

Gradient:

Time (min)	% A2 (or B2)
0	2.0
0.2	2.0
2.0	98.0
2.5	98.0
2.6	2.0
3.0	2.0

AutoPurification Conditions

Mobile Phase A:	water with modifier
Mobile Phase B:	acetonitrile with modifier
Modifier:	0.1% formic acid (low pH) 0.1% ammonium hydroxide (high pH)
Column:	XSelect CSH C_{18} OBD, 19 x 100 mm, 5 µm, part number 186005421

Flow Rate:	25 mL/min
Detection:	UV at 210 nm and 254 nm
MS Detection:	ESI+ mode
Scan Range:	100 to 1000 amu Mass-directed autopurification using FractionLynx using the mass of the target compound.
Data Management:	MassLynx CDS

Focused Gradient:

Time (min)	% A2 (or B2)
0	2.0
0.81	lower %B
10.0	upper %B
11.0	98.0
12.0	98.0
12.1	2.0
15.0	2.0

Lower and upper %B values vary based on the retention of the target compound.

Sample Preparation

Samples representative of synthetic reaction products were prepared by acetylating 100 mg of 18 different compounds in 2 mL of dichloromethane, using 2 mL of pyridine and 1 mL of acetic anhydride. Reactions were stirred for 1 hour at 45 °C, thoroughly evaporated to dryness and resolubilized accordingly in combinations of acetonitrile:methanol:water.

Results and Discussion

For the purposes of method development, pH switching can provide a significant impact in selectivity and separation for mixtures containing ionizable compounds.[1] However, routine switching of mobile-phase pH on traditional C_{18} columns can cause poor peak shape and retention time shifts for basic compounds.[2] The CSH column, however, maintains retention time and peak shape for basic compounds in low-ionic-strength mobile phases (i.e., formic acid) when routinely alternating between high and low pH, making it an ideal column for a screening protocol.[3] Additionally, the CSH column chemistry is available in UPLC and HPLC particle sizes, eliminating the need for method redevelopment when transferring from UPLC to a new column chemistry at the preparative HPLC scale.

Products from 18 different reactions were first analyzed using a fast 3-minute UPLC screening protocol at high and low pH, on an ACQUITY UPLC CSH C_{18} 2.1 x 30 mm, 1.7 µm Column. Out of the 18 reactions screened, 12 reactions were deemed successful based on identification of the target compound from the UPLC-MS screen (Table 1). These reactions were selected for purification of the compound of interest, using the high- or low-pH modifier giving the best separation, as identified from the screening protocol. Chromatograms from the screening of two different reactions are shown in Figure 1A (low pH) and 1B (high pH).

Reaction	Starting Compound	Modifier	Target Peak (min)	Target Mass (m/z)
1	Flurbiprofen	Low pH	1.38	366
2	Procatechuic acid	High pH	1.07	284
3	Aminosalicylic acid	Low pH	0.82	196
4	5-Hydroxyindole acetic acid	High pH	1.06	216
6	Epinepherine	Low pH	0.96	292
8	Ephedrine	High pH	1.01	190
9	Labetalol	Low pH	1.32	395
10	Nadolol	Low pH	0.99	478
11	Anthranilic acid	High pH	0.88	162
14	Loperamide	High pH	1.24	519
15	Indomethacin	Low pH	1.42	358
18	Mycophenolic acid	Low pH	1.20	302

Table 1. UPLC screening results for twelve successful reactions, listing the modifier resulting in the best separation condition on the CSH C_{18} column. The retention time and mass of the peak targeted for purification is also identified.

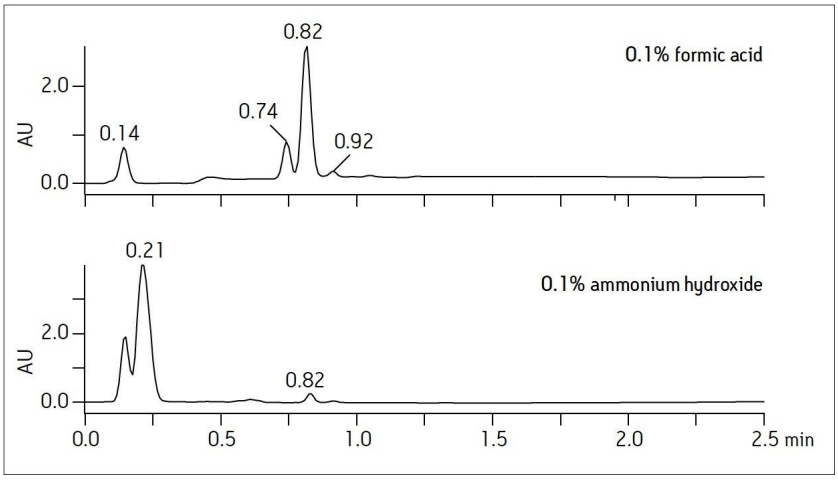


Figure 1A. UPLC screening results for aminosalicylic acid reaction products. 0.1% formic acid (low pH) modifier shows the best separation. Target peak identified at 0.82 minutes, m/z 196 (mono-acetylated product).

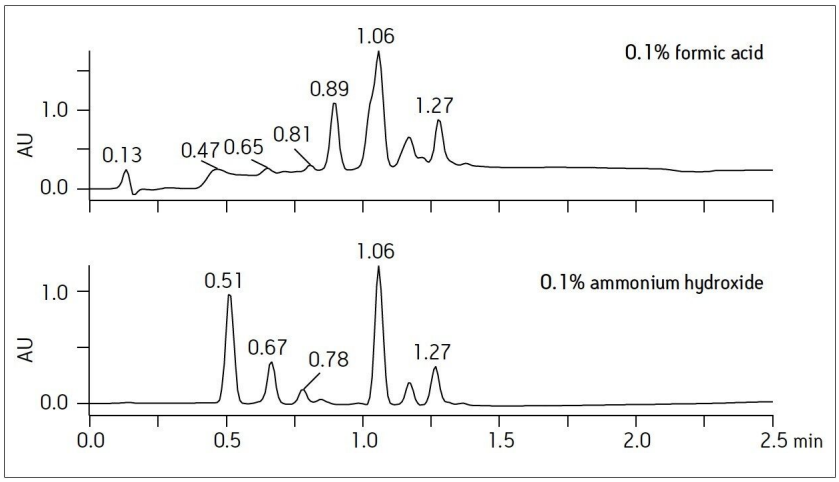


Figure 1B. Screening results for 5-hydroxyindoleacetic acid reaction products. 0.1% ammonium hydroxide (high pH) modifier shows the best separation. Target peak identified at 1.06 minutes, m/z 216 (mono-acetylated product).

Once the target compound for each reaction was identified from the UPLC screen, this information was used to calculate the focused gradient for preparative HPLC purification.[4,5] A focusing range of +/- 5% around the target peak was used. A 'generic' focused preparative HPLC gradient was created using the calculated

focused gradient segment time, with an overall runtime of 15 minutes including re-equilibration. By altering the original screening gradient to a shallower 'focused' gradient around the peak of interest, impurities before and after the target peak can be selectively pulled away to generate a cleaner purified sample (Figure 2). The use of focused gradients also minimizes preparative HPLC runtimes for faster sample purification throughput, as time is predominantly spent on the separation of the peak of interest rather than the entire sample.[6]

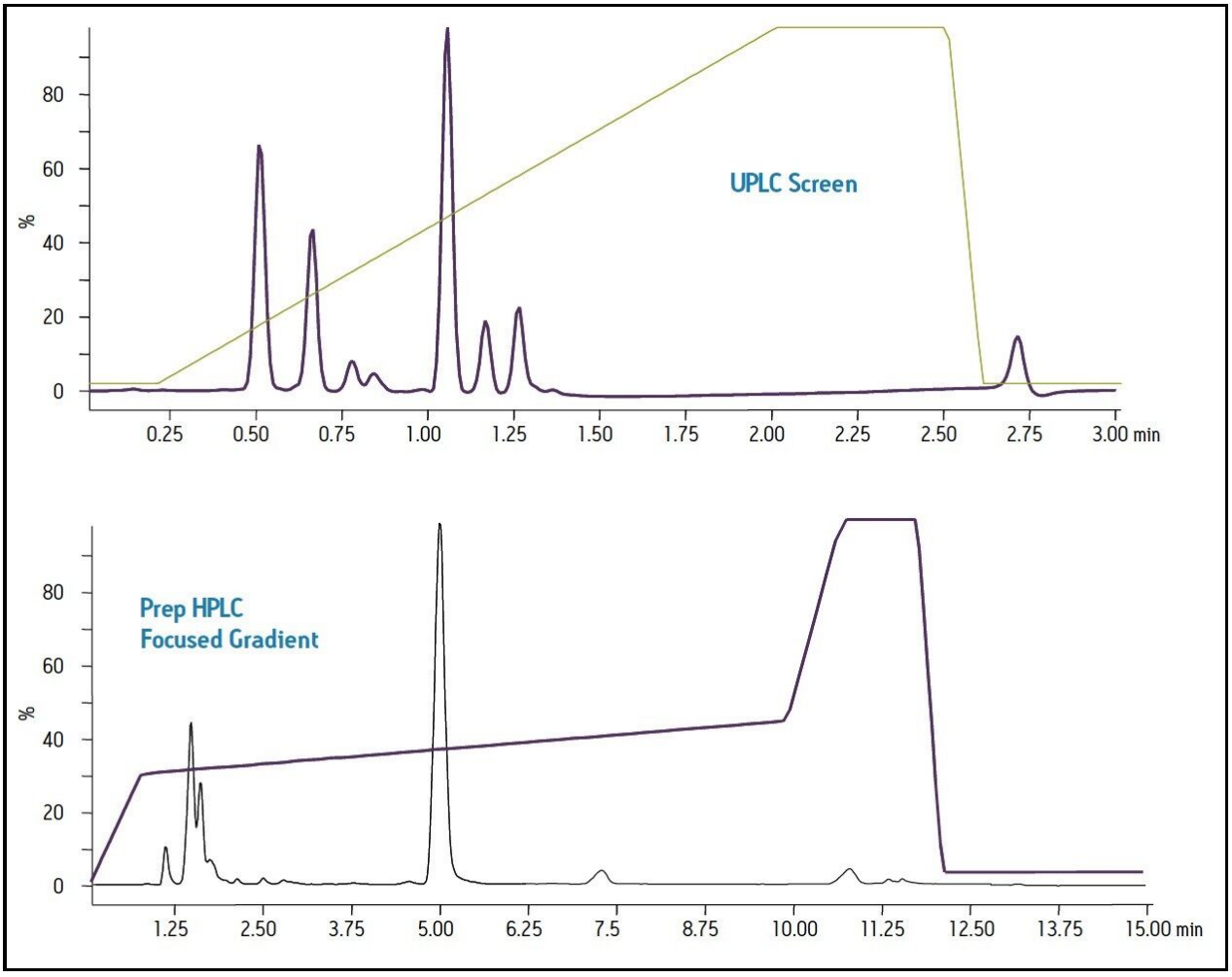


Figure 2. Gradient profiles for 5-hydroxyindoleacetic acid reaction products from the full UPLC screening gradient (2 to 98% B) and the preparative HPLC focused gradient (30.5% to 40.5% B).

Each of the twelve successful reactions was purified using a preparative HPLC focused gradient, entering the lower and upper %B limits calculated for each respective target compound. The compounds were purified using mass-directed autopurification of the target compound. The purified compound was reanalyzed using the UPLC screening method to confirm purity based on retention time, UV and mass spectral profiles.

Examples of method transfer from the UPLC screen to the preparative HPLC focused gradient are shown in Figure 3 (low pH) and Figure 4 (high pH). In Figure 3, the reaction products of labetalol acetylation are shown. While the UPLC screening chromatogram at low pH shows one major peak, the MS spectrum of this peak indicates two co-eluting products. The gradient is focused around the target peak of 1.32 minutes and the target compound is purified using the preparative HPLC focused gradient. While there is still some apparent co-elution, the shallower focused gradient around the peak of interest helps to separate the compound of interest (*m/z* 395) into a discrete peak, which is collected using mass-directed autopurification. The purified compound is then re-run using the UPLC screening method, yielding a product with demonstrated purity.

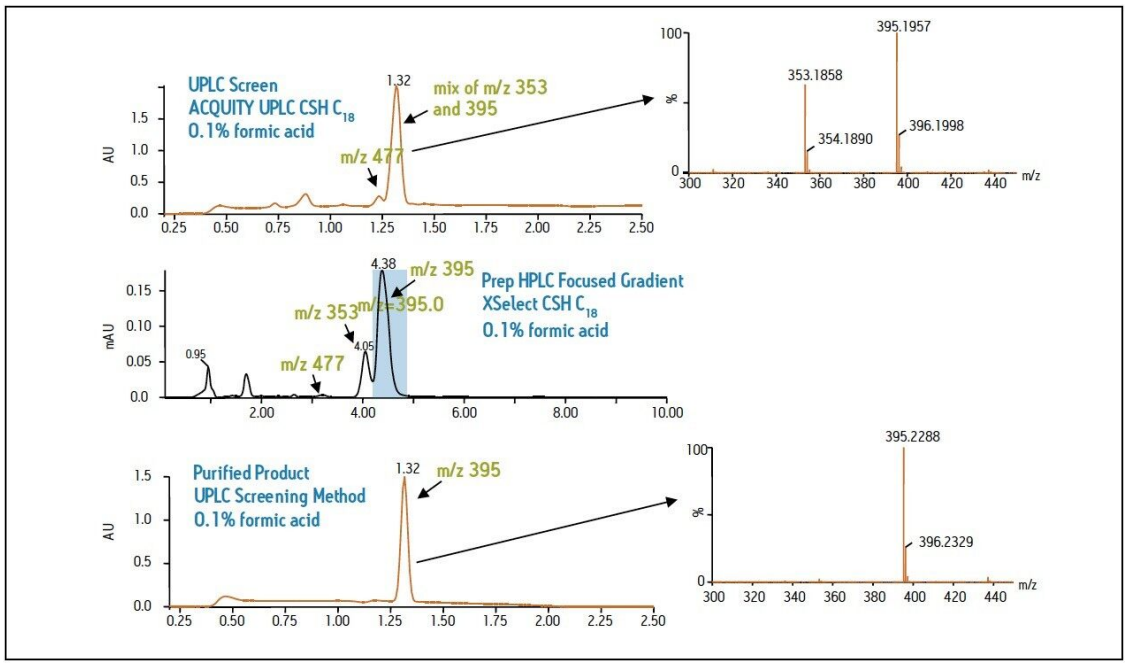


Figure 3. Labetalol reaction products: UPLC screen result with target peak at 1.32 minutes with m/z 395 (mono-acetylated product), transferred to preparative HPLC using a focused-gradient (50 mg loading). Mass-directed purification of the targeted m/z 395 peak results in a pure product, confirmed by running the purified product using the UPLC screening protocol.

In a different example (Figure 4), the UPLC screening results reveal that the acetylation products of 5-hydroxyindole acetic acid are best separated at high pH. The target peak is identified at 1.06 minutes, and the method is transferred to the preparative HPLC using a focused gradient around this peak. The preparative HPLC chromatogram shows the impurities pulled away from the peak of interest, allowing for greater loading and more effective purification of the target compound.

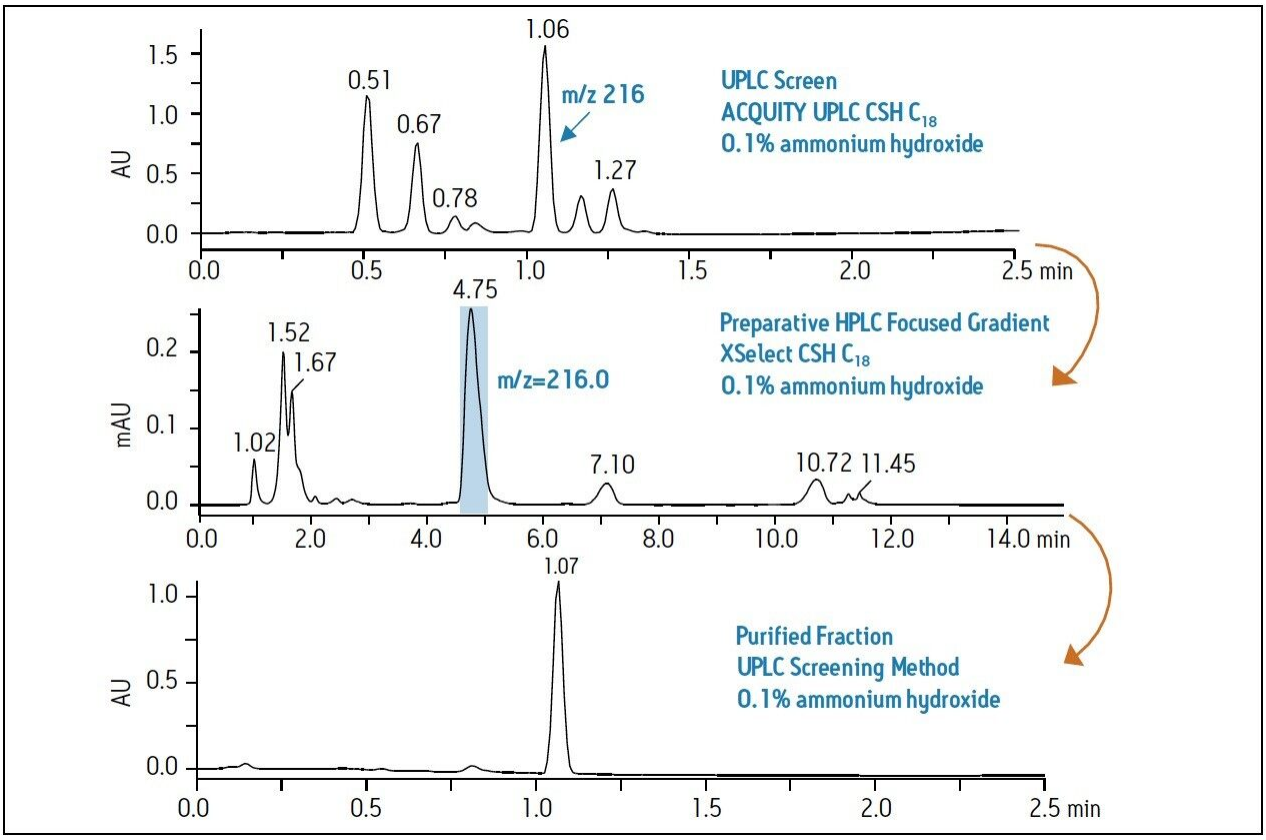


Figure 4. 5-hydroxyindoleacetic acid reaction products: UPLC screen result with target peak at 1.06 minutes with m/z 216 (mono-acetylated product), transferred to preparative HPLC using a focused-gradient (50 mg loading). Mass-directed purification of the targeted m/z 216 peak results in a pure product, confirmed by analyzing the purified product using the UPLC screening protocol.

Conclusion

- A 3-minute UPLC-MS method to reliably screen high and low pH for quick determination of optimal separations conditions was developed using an ACQUITY UPLC CSH C_{18}, 2.1 x 30 mm, 1.7 µm Column.
- The time-consuming need to fully redevelop a method when scaling a separation from UPLC to preparative HPLC was eliminated by using the same CSH C_{18} column stationary phase, greatly simplifying method transfer.
- The use of focused gradients shortens preparative runtimes and increases sample throughput by selectively focusing on the separation around the peak of interest, rather than all components in the

mixture. In addition, focusing the separation around the peak of interest can better resolve near-eluting impurities, resulting in increased loading capability and cleaner purified fractions.

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