An Undergraduate's Approach to Method Development: Chromatography Lessons Learned through Experimentation

Application Note PHA0512

The data for this application note was a collaboration between Gilson, Inc. and Amanda Bayer, University of Wisconsin, Madison, WI, USA, during a summer internship at Gilson, Inc. in Middleton, WI

Keywords

PLC 2020, Purification, Preparative Chromatography, Reversed Phase Chromatography, Method Development, Separation Science, Pharmaceuticals

Introduction

Reversed phase chromatography accounts for approximately 75% of the chromatography separations performed today, creating the need for an undergraduate in the field of chemistry to fully understand this specific chromatographic technique prior to entering the pharmaceutical industry. Turning theory into practice is essential to allow for a complete understanding of reversed phase chromatography fundamentals and making a student's transition into industry most successful. As part of a summer internship, an undergraduate student was given a variety of common pharmaceutical compounds and given the challenge of finding a gradient that would separate two or more of the compounds with good peak shape. This application note details the process the student went through in determining gradient conditions and sample composition using beginner chromatography knowledge.

Figure 1. Gilson PLC 2020 Personal Purification System.

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Materials & Methods

Samples & Solvents

Note: All samples and solvents used were of HPLC grade.

- Acetaminophen
- Aspirin
- Ibuprofen
- Naproxen
- NanoPure Water
- Methanol
- Formic Acid
- Sodium Hydroxide
    - 50% solution was prepared with NanoPure water

Apparatus

- Gilson PLC 2020
    - 50 SC Pump Heads
    - 5 mL Sample Loop
    - 0.2 mm Pathlength Detector Flow Cell
- Phenomenex Luna C18(2), 5 micron, 50x21.2 mm

General HPLC Parameters Used throughout Method Development

- Mobile Phase
  A: 0.02% Formic Acid in Water
  B: Methanol
- Flow Rate: 25 mL/min
- Detector Wavelength
  Primary: 220 nm
  Monitor: 254 nm

Results

The student was given four compounds (Table 1) consisting of Acetaminophen, Aspirin, Ibuprofen, and Naproxen. A common reversed phase starting gradient using 0.02% formic acid in water with methanol was used as a starting point (Table 2, Figure 2). All modifications performed were based on the student's understanding of separation chemistry, with the goal of producing a gradient that separates at least two of the compounds with good peak shape. The following figures and tables walk through the timeline of modifications made during the course of method development.

Table 1. Initial Sample Preparation.

Compound	Acetaminophen	Aspirin	Ibuprofen	Naproxen
Diluent	70:30 MPA:MPB*	70:30 MPA:MPB	70:30 MPA:MPB	70:30 MPA:MPB
Concentration	2.5 mg/mL	2.5 mg/mL	2.5 mg/mL	2.5 mg/mL
Soluble	Yes	Yes	No	No
Diluent			70:30 MPA:MPB	70:30 MPA:MPB
Concentration			2 mg/mL	2 mg/mL
Soluble			No	No
Diluent			70:30 MPA:MPB	70:30 MPA:MPB
Concentration			1.5 mg/mL	1.5 mg/mL
Soluble			No	No
Diluent			70:30 MPA:MPB pH = 14	Methanol
Concentration			1.5 mg/mL	2.5 mg/mL
Soluble			Yes	Yes
Retention Time	0.815 min Figure 3A	3.721 min Figure 3B	13.724 min Figure 3C	1.715 min Figure 3D

* Note: MPA = 0.02% Formic Acid in Water, MPB = Methanol

Table 2. Initial Gradient Conditions.

Step No.	Time (min)	%A	%B
1	0 – 2	70	30
2	12 – 13	30	70
3	14 – 15	70	30

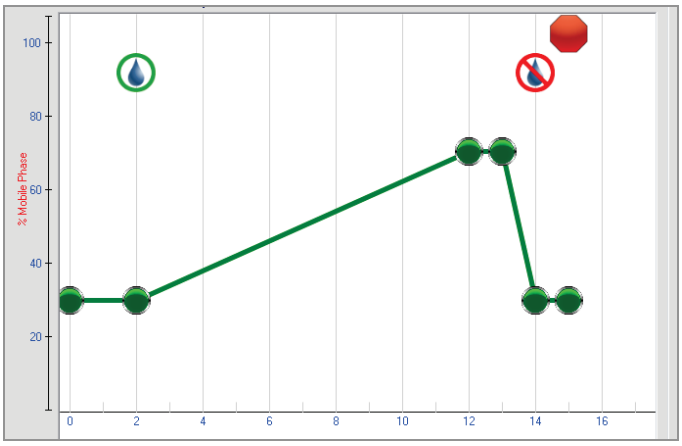

Figure 2. PLC 2020 Initial Gradient Conditions.

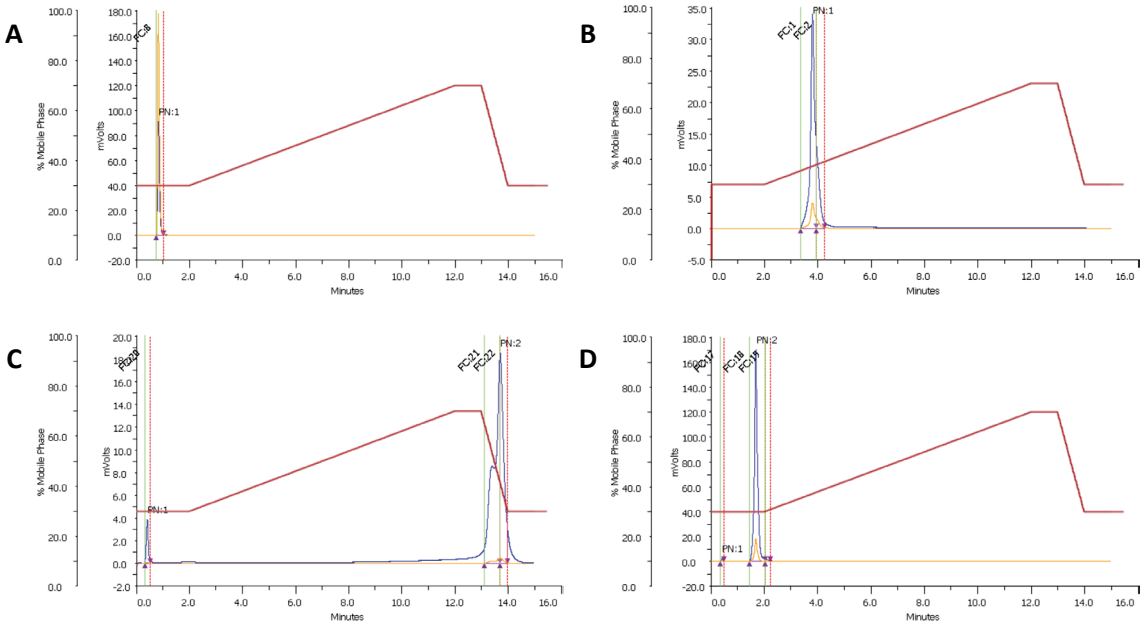

Figure 3. Injections of Individual Compounds with Initial Gradient.
A) Acetaminophen **B)** Aspirin **C)** Ibuprofen **D)** Naproxen

Following initial chromatography injections, modifications were made to optimize elution time and peak shape. In modification #1 the gradient was adjusted to bring the late eluting Ibuprofen peak forward (Table 3, Figures 4, 5).

Table 3. Gradient Conditions, Modification #1

Step No.	Time (min)	%A	%B
1	0 – 2	70	30
2	6	54	46
3	8 – 11	30	70
4	11.6 – 12.6	70	30

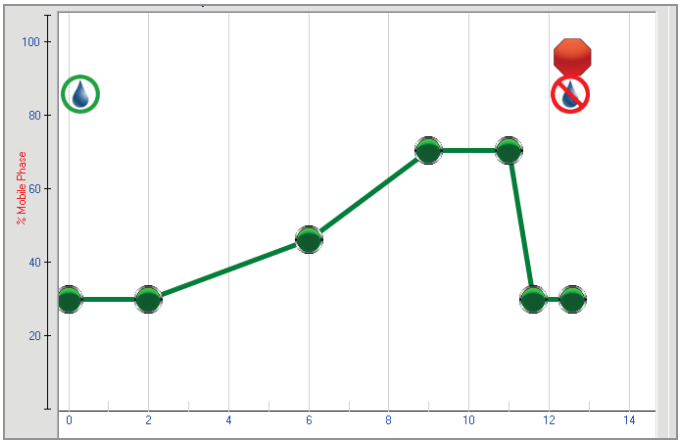

Figure 4. PLC 2020 Modification #1 Gradient Conditions.

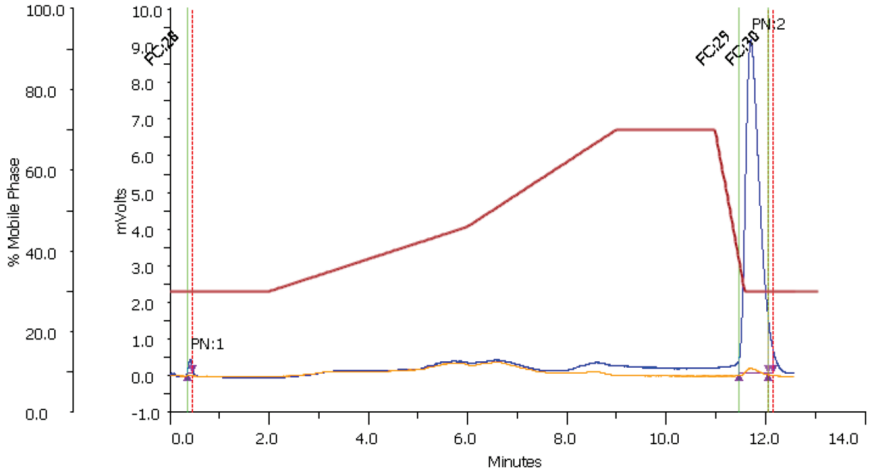

Figure 5. Ibuprofen Injection Using Gradient Modification #1 (RT = 11.721).

Solutions were combined to result in a sample of 5 mg/mL of each compound and injected using the gradient from modification #1. Chromatography results show an unidentified peak overlapping with the Acetaminophen peak. Also, the Naproxen peak was off-scale (Figure 6).

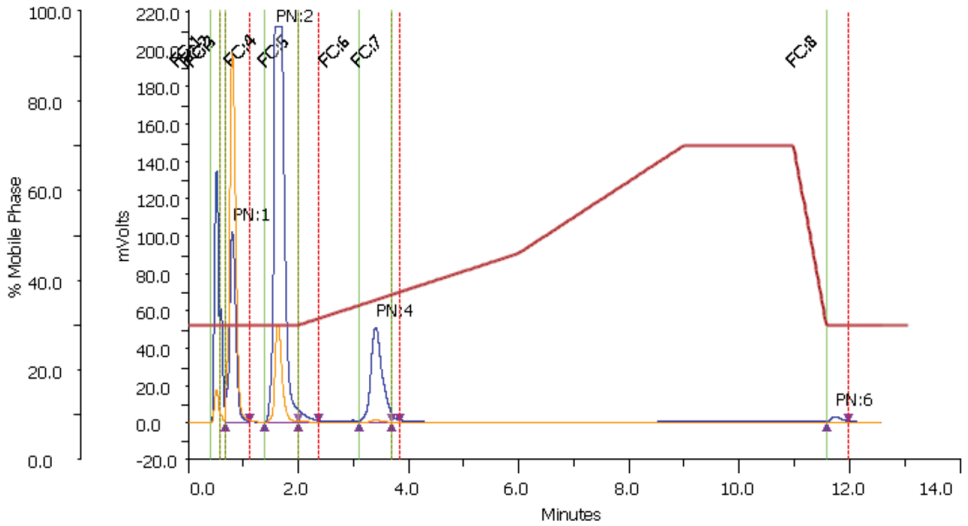

Figure 6. Combination Injection (5 mg/mL) Using Gradient Modification #1.
(Peak 1: Acetaminophen, Peak 2: Naproxen, Peak 4: Aspirin, Peak 6: Ibuprofen)

The concentration of the solution was then decreased by 0.5 mg/mL increments until the Naproxen peak was on-scale. The final concentration was 3 mg/mL. The gradient was then adjusted (modification #2, Table 4, Figure 7) to increase resolution between the early eluting peaks.

Table 4. Gradient Conditions, Modification #2.

Step No.	Time (min)	%A	%B
1	0	100	0
2	1.5	90	10
3	6	70	30
4	9 – 11	30	70
5	11.6 – 12.6	70	30

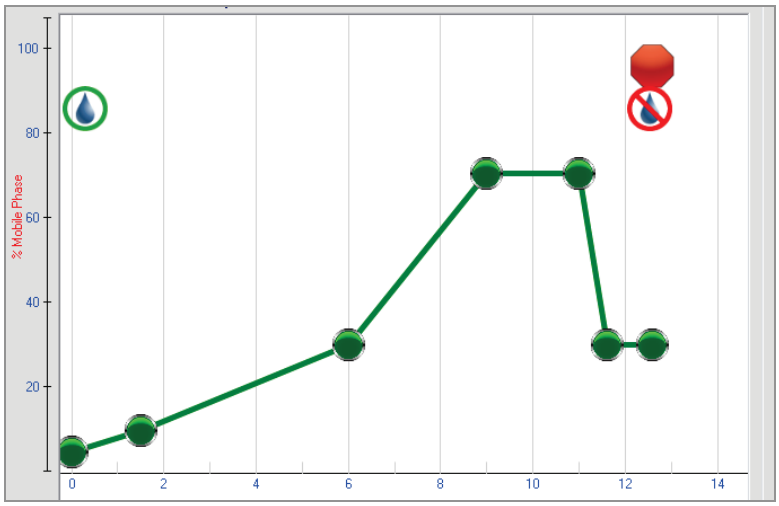

Figure 7. PLC 2020 Modification #2Gradient Conditions.

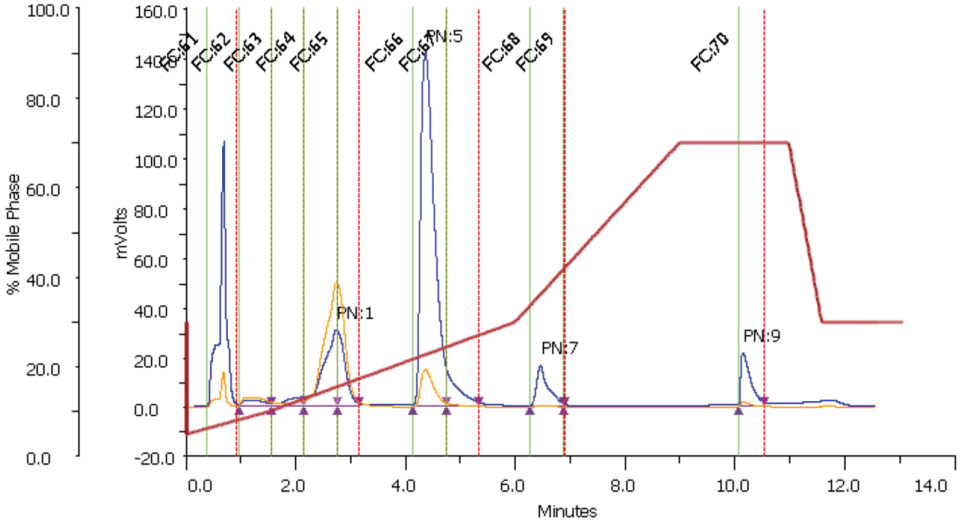

Figure 8. Solution Injection (3 mg/mL naproxen, 5 mg/mL all others)
Using Gradient Modification #2.
(Peak 1: Acetaminophen, Peak 5: Naproxen, Peak 7: Aspirin, Peak 9: Ibuprofen)

It was decided that Ibuprofen would be removed from the test solution due to its low response at the wavelengths used, late elution time, and need for a modified diluent. Removing Ibuprofen allowed all solution compounds to elute in less than 5 minutes with a few additional gradient adjustments (modification #3, Table 5, Figure 9).

Table 5. Gradient Conditions, Modification #3.

Step No.	Time (min)	%A	%B
1	0	100	0
2	1.5	90	10
3	6	70	30
4	9 – 9.55	30	70
5	10 – 11.5	70	30

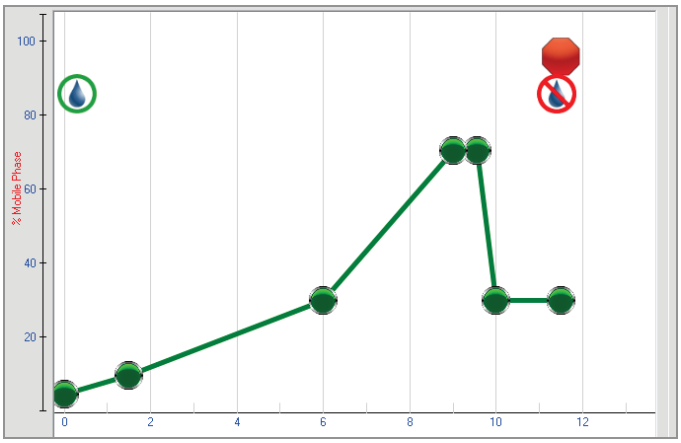

Figure 9. PLC 2020 Modification #3 Gradient Conditions.

Extraneous peaks were seen in a subsequent injection of the remaining compounds in solution. Pairs of the compounds were injected to look for possible interaction. The combination of Acetaminophen and Aspirin produced extra peaks corresponding to those detected in the full compound solution (Figure 10).

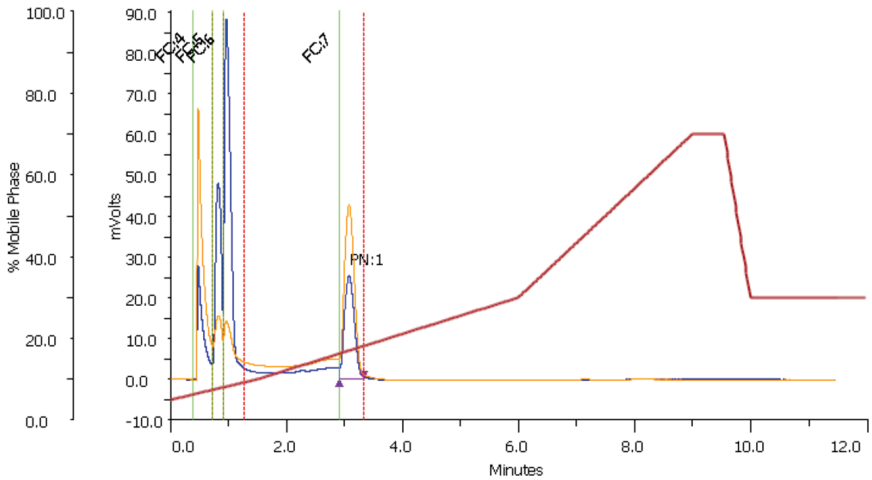

Figure 10. Injection of Acetaminophen and Aspirin Using Gradient Modification #3.

Removing Acetaminophen from the compound solution eliminated the interaction. The resulting compound solution now contained only Naproxen and Aspirin. A final gradient modification was made (modification #4, Table 6, Figure 11), and a final injection of the Naproxen and Aspirin solution resulted in good resolution and peak shape for both compounds (Figure 12).

Table 6. Gradient Conditions, Modification #4.

Step No.	Time (min)	%A	%B
1	0	100	0
2	1.5	90	10
3	6	70	30
4	9 – 9.55	30	70
5	10 – 11.5	70	30

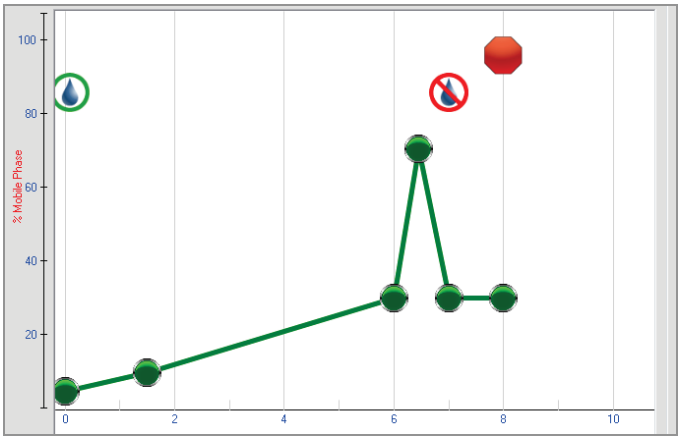

Figure 11. PLC 2020 Modification #4 Gradient Conditions.

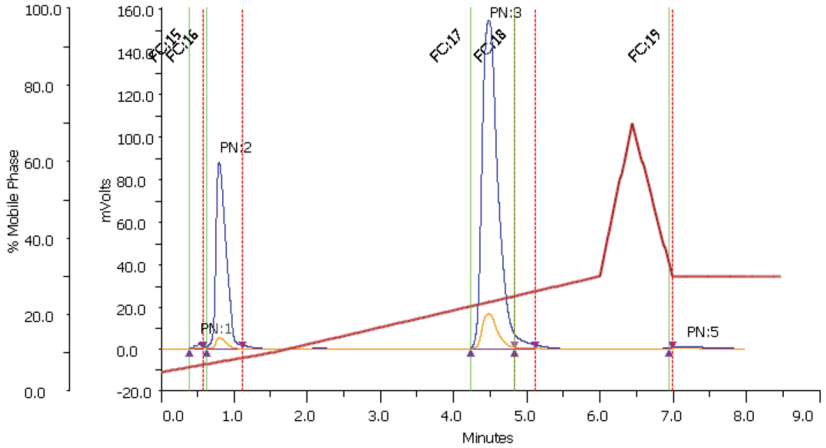

Figure 12. Injection of Final Compound Solution (3 mg/mL Naproxen, 5 mg/mL Aspirin) Using Gradient Modification #4.

Summary

Through using the simple touch screen PLC 2020 software, a beginner chromatographer was able to navigate through a method development problem using previous academic knowledge. The student used separation chemistry to adjust and determine mobile phase composition modifications #1-4 to conclude with well-resolved, Gaussian peaks.

Though the larger goal of separation was achieved, some of the details of the method development process could be further refined to improve chromatography. Further adjustments to the gradient could be made, including bringing the gradient back to starting conditions at the end of the run and removing the acid modifier in the mobile phase, as the Naproxen solubility is pH dependent. An example of a suggested gradient modification to move forward with in further experimentation is found below (Figure 13). The organic content of the sample diluent should also be adjusted as starting conditions change. Sometimes obvious principles to an experienced chromatographer may not be recognized by someone new to the technique.

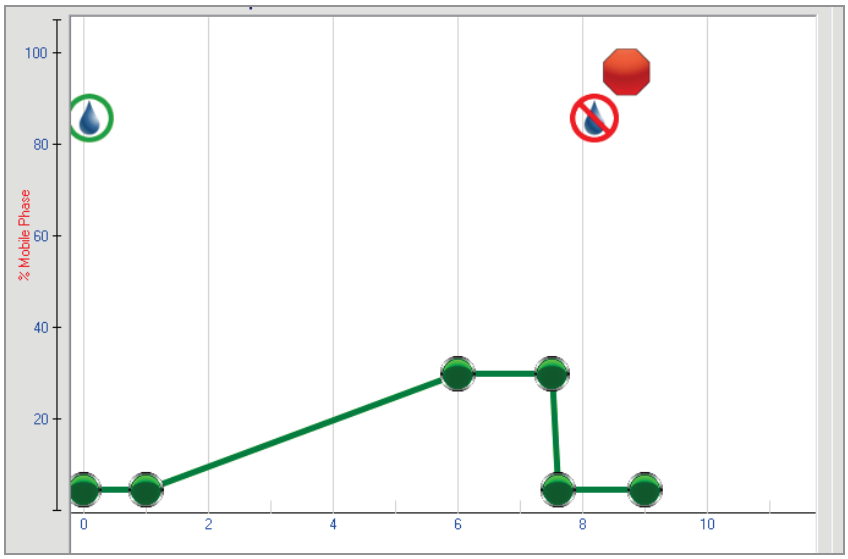

Figure 13. Proposed PLC 2020 Modification #5 Gradient Conditions.