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Calibration of analytical HPLC to generate preparative LC gradients for peptide purification

Jack Silver

Teledyne ISCO, United States

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Abstract

Preparative LC (liquid chromatography) is widely used to purify synthesized peptides. One bottleneck in the purification process is method development. Significant time can be required to produce an efficient preparative purification method that resolves the desired peptide from impurities and minimizes both time and solvent usage. This work describes a simple method of calibrating analytical HPLC systems to match the preparative LC system using the existing scouting gradients typically employed by a research group. After the calibration is complete, the determined delay volume is applied to the scouting gradient. This delay volume encompasses any dwell volumes, column volumes, mixing volumes, solvent mis-proportioning, and other corrections that are needed to match the analytical system to the preparative system. After the calibration is complete, the user only needs to enter the retention time of the desired compound from the analytical HPLC scouting run to calculate a preparative method. Although the calculated gradient is designed to run over 12 minutes with targeted peptides eluting at ~6 minutes, other gradient lengths may be run.

Prep System Calibration

This requires three steps:

1. Determine the dwell volume (V_{Dp}). This only needs to be done once. Replace one solvent with a solvent that absorbs UV light. Acetone is useful since it is miscible with water and easily washed from the system. Replace the column with a union. Run isocratically with no absorbing solvent, then program a step gradient. Note the delay in absorbance from the step and multiply by the flow rate to obtain the dwell volume.

2. Determine the column volume (V_{Cp}). Do this once per column size and chemistry used; replacement columns of the same type will have the same column volume. For example, all RediSep Prep 20 x 150 mm C18 columns will have the same column volume. Run the column with 10% organic, and inject a small amount of sodium iodide or sodium nitrate while monitoring 215 nm. Note the peak elution time and multiply by the flow rate to obtain the column volume. Steps 1 and 2 allow the calculated gradient to be adjusted for the delays of the preparative system.

3. Set the elution time for the model compound using an isocratic run. Use the same solvent and modifiers typically run on the analytical system. Adjust the mobile phase composition to elute the compound at the desired time for the preparative runs. Model compounds used include ethyl paraben, phenacetin, and N-benzylbenzamide. These were chosen because they elute at ~50% organic solvent. This step sets the retention time for the column used.

Analytical System Calibration

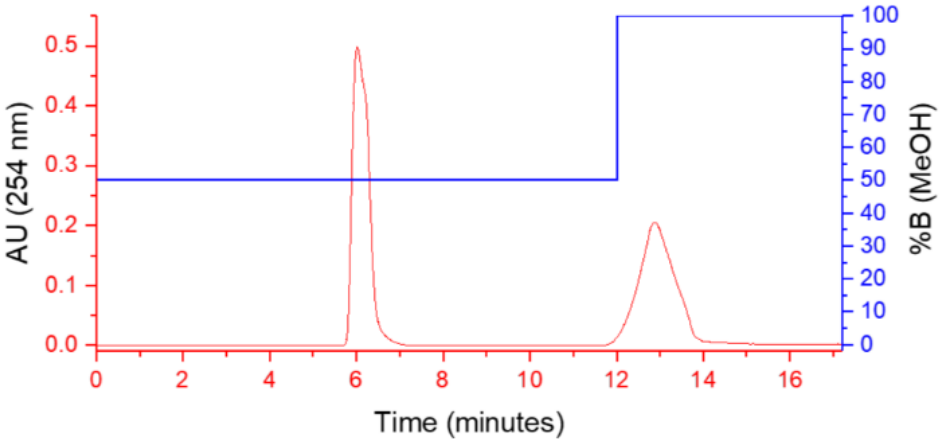

Figure 1: Calibration of a 20 x 150 mm RediSep Prep C18 column (PN 692203826) with phenacetin and N-benzylbenzamide in methanol on an ACCQPrep HP125 (PN 685230035). Model compound (phenacetin) eluted in ~6 minutes at 50% B solvent.

This only requires running the model compound with the scouting gradient used to evaluate synthesized compounds. This gradient is typically 5 or 10% to 100% organic with no isocratic hold at the start. Use the same solvent system, including modifiers, as used to calibrate the preparative system. Use columns with the same packing for preparative and analytical runs. After calibration, the programmed gradient time for the calibration compound is calculated using the equations below.

$Ma = (\%B_{Ea} - \%B_{Sa})/L_a$

$P = (\%B_{I} - \%B_{Sa})/M_a$

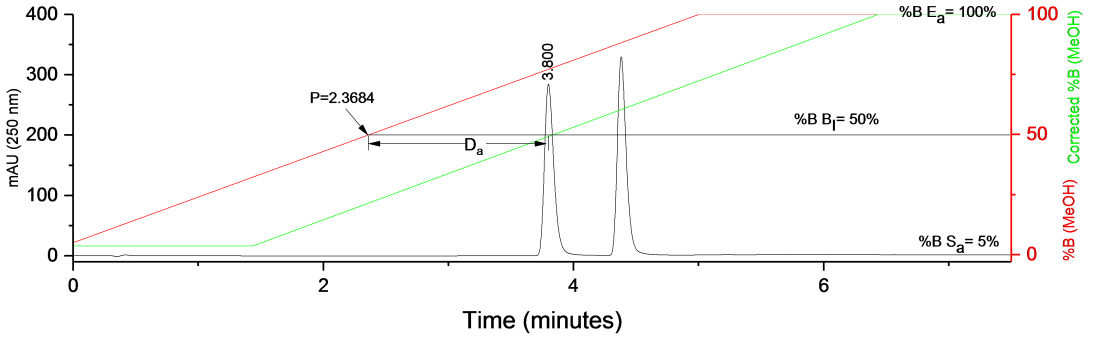

Figure 2: Sample from prep calibration run (Figure 1) on an Agilent UHPLC using an experimental 2x50 mm RediSep Prep C18 column in water/methanol. The gradient was 5-100% B over 5 minutes. The peak eluting at 3.800 minutes (phenacetin) is deemed to elute at 50% methanol.

Focused Gradient Calculation

This requires four steps:

1. Run the compound to be purified on the analytical system using the same gradient as the initial calibration.
2. Using the calculated value Da, determine the actual %B which elutes the compound.
3. Set a focused gradient encompassing the calculated %B.
4. Correct the gradient for the dwell and column volumes of the preparative system

$\%B_{Corr} = (T_{Ea} - D_a) * M_a + \%B_{Sa}$

The corrected solvent composition for the desired compound is $\%B_{Corr}$; T_{Ea} = the elution time for the desired compound in the analytical run. The other terms are the same as the earlier equations.

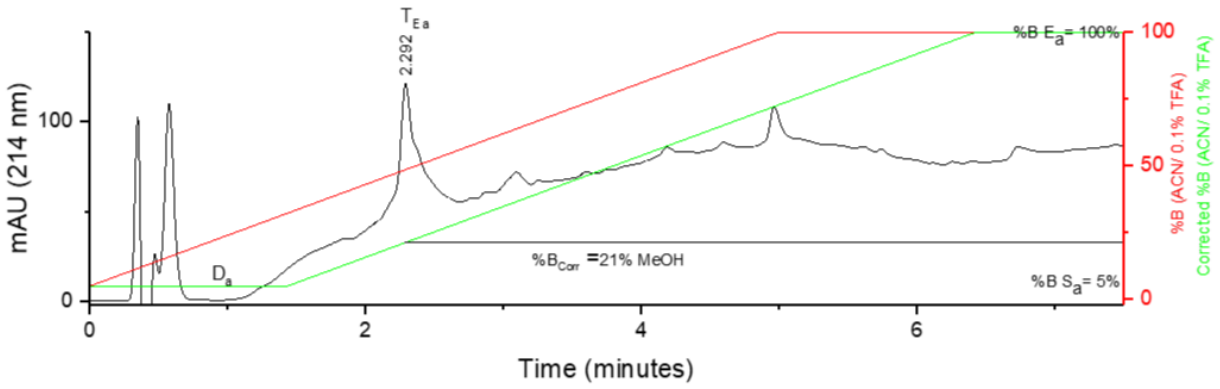

Figure 3: Analytical run of Thymosin (H2N-SDAAVDTSSEITTKDLKEKKEVVEEAEN-OH, MW 3066) using the same gradient and column as Figure 2. The solvent system was water/acetonitrile, both containing 0.1% TFA. Thymosin eluted at 2.292 minutes.

The retention of thymosin is very different from the calibration compound and thus serves as a useful example. For this example:

$\%B_{Corr} = (2.292–1.43216) * 19 + 5 = 21.3\ \%B$

Set a focused gradient with the desired range (R), centered around this number—usually a range of 10% to 20%.

$D_p = (V_{Dp} + V_{Cp})/F_p$

$\Delta\%B = R/L_p * D_p$

The final steps to calculating the gradient involve correcting the focused gradient for the preparative system. For the equations above, D_p, the preparative system delay, is determined by adding the prep system dwell volume (V_{Dp}) to the prep column volume (V_{Cp}) and dividing by the prep flow rate. The amount to increase the strong solvent concentration (Δ%B) is calculated by dividing the range (R) by the prep gradient length (L_p) then multiplying by D_p. For an ACCQPrep 125, V_{Dp} = 7.65 mL (with 5 mL loop); V_{Cp} = 24.81 mL (C18 20x150 mm RediSep Prep column), run at 18.9 mL/min. The chosen range (R) is 10%, with a length (L_p) of 12 minutes. D_p = (7.65 + 24.81)/18.9 = 1.7174 min; Δ%B = 10/12 *1.7174 = 1.4312% The final gradient is 22.8 % ±5, or 18 to 28% methanol.

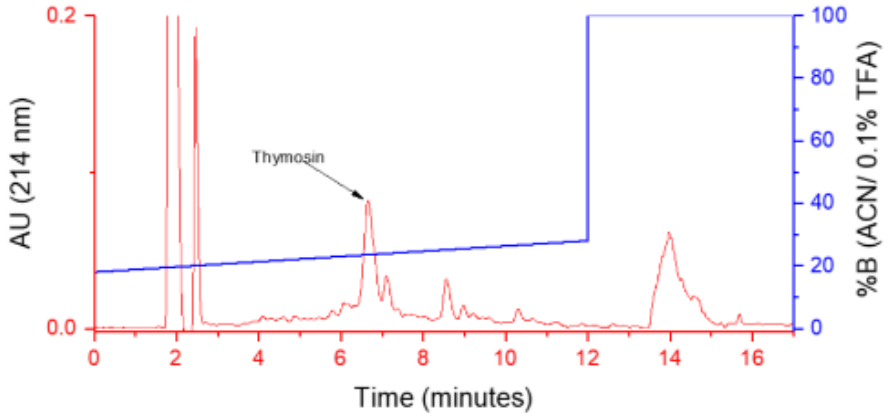

Figure 4: Thymosin (12 mg dissolved in 0.2 mL DMSO) run on a RediSep Prep C18 column in water/acetonitrile (both containing 0.1% TFA) using the calibration described in this poster eluted at the expected time. Although the calibration was performed in methanol with no modifiers, the gradient was correctly calculated when the compound was eluted from both the analytical and preparative system using acetonitrile modified with TFA.