Designing Purification Methods for Hydrophobic Peptides

Solubility Trials Can Be the Key

Hydrophobic peptides pose a challenge in developing purifications. They are often difficult to dissolve, with low solubility in pure aqueous or organic solvents, but soluble at intermediate compositions. Dissolution studies can provide valuable information for chromatography. Find a solvent mixture that dissolves the peptide. Dilute with the aqueous component, but not so much as to cause the peptide to fall out of solution. Then use that as a starting point for the chromatographic mobile phase.

Table I

Step 1. Strong (Organic) Component	Step 2. Buffer	Step 3. Weak (Aqueous) Component	Success?
CHCl3	TEAA pH6	acetone	**NO**
CHCl3 + *n*-propanol	TEAA	water	**NO**
THF + *n*-propanol	TEAA	water	**NO**
100µL *n*-propanol + 100µL THF	100µL HOAc	100µL water	**YES**
200µL *n*-propanol	100µL HOAc	100µL water	**YES**
200µL *n*-propanol	10µL HOAC	100µL 5%HOAc in water	**YES**

Direct dissolution of hydrophobic peptides in aqueous solvent mixtures can be slow. Frequently a peptide will appear to be insoluble when it is actually the kinetics of dissolution that are causing difficulty. If you encounter apparent insolubility problems, try the following procedure with a selection of likely solvents and trial samples of the peptide.

(1) Always add the pure solvent, i.e., the organic component, first. This overcomes wetting problems and brings hydrophobic parts of the peptide into the liquid phase.

(2) Next add concentrated buffer components. This sets the pH, which adjusts the charges on ionic amino acids.

(3) Finally, add the aqueous portion of the mixture. In many cases the peptide will not dissolve until this final step, but a peptide that appeared insoluble will dissolve rapidly when these steps are followed. Here is an example:

A synthetic peptide provided by Dr. Don Diamond (City of Hope National Medical Center, Duarte, CA) was known to contain 25 amino acid residues and two attached fatty acids. Approximately one milligram of peptide was added to each of six 6 x 50 mm test tubes, and the dissolution sequences in Table I were attempted.

Based on the results of Table I, 10 mg of the peptide was solubilized by adding 3.6 mL of *n*-propanol, then 200 µL of glacial acetic acid, and finally 0.6 mL of 5% acetic acid in water. The peptide does not dissolve until the 5% acetic acid is added. The resulting solution is 2.27 mg/mL peptide in 86% *n*-propanol/5% acetic acid. When an aliquot was diluted 8/1 with 0.5% acetic acid, the peptide remained in solution.

Initial attempts at chromatography using a gradient from 5% to 85% *n*-propanol in 0.5% HOAc on a C4 column (Vydac 214TP54) resulted in no elution of peptide (as A_{280}). The same procedure on a diphenyl column (Vydac 219TP54) produced the chromatogram of Figure 1.

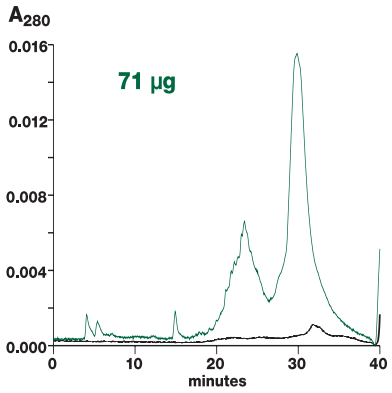

Figure 1. Initial trial chromatogram of lipid peptide. Column: Vydac 219TP54 phenyl reversed-phase, 4.6mmID x 250mmL. Sample: 250 µL of 8:1 diluted peptide solution = 71 µg. Flow rate: 0.75 mL/min. Mobile phase: A = 5% n-propanol/0.5% HOAc. B = 85% n-propanol/0.5% HOAc. Gradient: 0% to 100% B in 30 minutes. The black trace is a post-run blank.

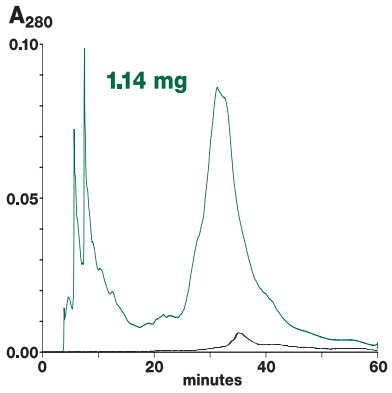

Figure 2. Initial prep chromatogram of lipid peptide. Column: Same as Figure 1. Sample: 1.5 mL of 3:1 diluted peptide solution = 1.14 mg. Three 500 µL injections at 1.5 minute intervals. Flow rate: 0.75 mL/min. Mobile phase: A = 29% n-propanol/0.5% HOAc. B = 61% n-propanol/0.5% HOAc. Gradient: Hold 100% A for 6 minutes. Then, 0% to 100% B in 30 minutes. Black trace is post-run blank.

Based on these results, a much larger sample of peptide was applied to the diphenyl column equilibrated at 29% *n*-propanol in 0.5% HOAc. This produced the chromatogram of Figure 2, the starting point for a preparative method. Further development might include adding ACN to the mobile phase, trying Vydac's 259VHP polymer reversed-phase column, and ion-exchange in 50% *n*-propanol/acetic acid as an orthogonal separation step.

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Developing a Method for Analysis

To monitor success of purification, a high-resolution analytical method is also needed. The major concern for preparative chromatography was to maintain solubility and avoid irreversible aggregation of high concentrations of sample on the chromatographic column. Under analytical conditions, however, solubility and aggregation should be less problematic. Lower sample loads allow more latitude in choice of solvents. The primary objective: to maximize resolution. In addition, using different chromatographic conditions, for example TFA as the modifier vs. acetic acid used for preparative runs, can provide greater assurance of detecting coeluting impurities.

Higher viscosity and slower diffusion generally make gradients of solvents such as *n*-propanol less suitable for analytical chromatography than methanol or acetonitrile. In this case, first attempts at analytical chromatography were made substituting acetonitrile in the eluting solvent for reversed phase on the Vydac 219TP diphenyl column. Figure 3 shows analytical chromatograms with gradients of acetonitrile alone as well as 50% blends of acetonitrile with isopropanol and *n*-propanol. The surprising result is that the peptide appeared to elute more rapidly with acetonitrile alone than in combination with the solvent that was known to favor dissolution. At first glance, a gradient of acetonitrile in 0.1% TFA appeared to be suitable for analytical chromatography.

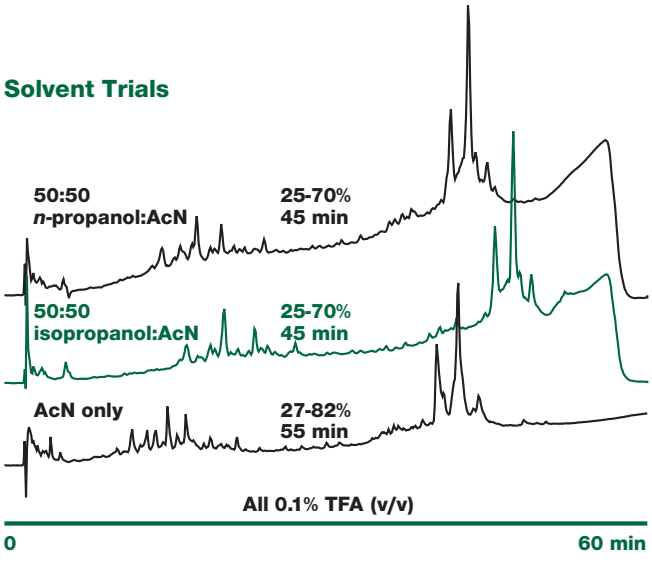

Figure 3. Trial chromatograms for analytical reversed-phase separation of lipid peptide with different eluting solvents. In each case, gradients were run with solvents in the proportions indicated, 0.1% TFA (v/v), and water as the aqeous mobile-phase component. Column: Vydac 219TP54 (diphenyl, 4.6mmID x 250mmL). Flow rate: 1.5 mL/min. Temperature: 45° C.

Not so, however, as revealed by post-run blank chromatograms, an important check on chromatography of any difficult-to-dissolve material. When chromatography was done with acetonitrile alone (Figure 4) the column showed significant "memory", in the form of ghost peaks appearing in the eluate with no sample applied.

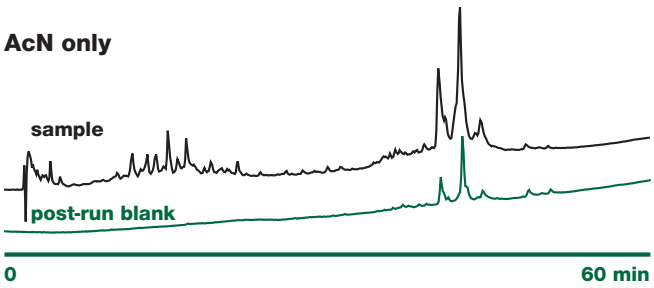

Figure 4. Examination of post-run blank for chromatogram with acetonitrile only. Conditions as in Figure 3.

An important lesson for analytical method development: Always perform post-run blanks to check for memory effects.

With a 50:50 acetonitrile:*n*-propanol blend as the eluting solvent, the post-run blank was clean (Figure 5), demonstrating the importance of the stronger solvent in this case for preventing aggregation even at analytical concentrations. Use of *n*-propanol as the blended solvent appeared to have a slight edge in resolution over isopropanol for this particular peptide and was the preferred choice for the analytical method.

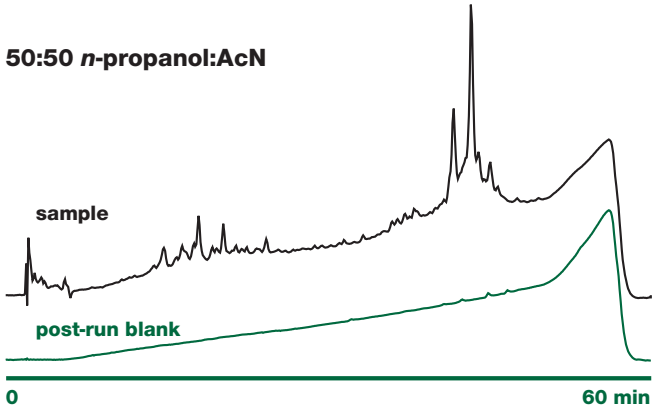

Figure 5. Examination of post-run blank for chromatogram run with 50:50 acetonitrile:n-propanol. Conditions as in Figure 3.

ORDERING INFORMATION:

Cat. No.	Description
	Column used in this work:
219TP54	Diphenyl Reversed-Phase Column, 300Å, 5µm, 4.6mmID x 250mmL
	Other Vydac 300Å reversed-phase columns:
214TP54	C4, 300Å, 5µm, 4.6mmID x 250mmL
208TP54	C8, 300Å, 5µm, 4.6mmID x 250mmL
218TP54	C18, Polymeric, 300Å, 5µm, 4.6mmID x 250mmL
238TP54	C18, Monomeric, 300Å, 5µm, 4.6mmID x 250mmL
259VHP5415	Polymer RP, 300Å, 5µm, 4.6mmID x 150mmL

Other analytical and preparative column dimensions available upon request.

To place an order, call The Nest Group 800.347.6378 your local Vydac distributor.