Preparative Scale Purification of Leuprolide by Concentration Overload

Application Note

Authors

Stephen Ball, Keeley Mapp and Linda Lloyd
Agilent Technologies, Inc.

Introduction

The use of peptides as biopharmaceuticals is one of the major growth areas in current drug development. The method of manufacture can be solid phase synthesis, solution phase synthesis or the use of recombinant/biotechnology. For API production, the choice of the peptide manufacturing method will depend on the peptide properties, amino acid sequence, the number of amino acids, and the amount of final product required. Many of the peptides currently being investigated are of the order of 8 to 15 amino acids in length and these are most suited to the solid phase synthesis route. There have been major advances in methods and resins used for solid phase synthesis which has resulted in an increase in the purity of the crude peptides but for biopharmaceutical applications, purification is still required. When developing purification methods, consideration must be given to the amount of peptide required, not only for clinical studies but also at API production stage, so that the production method is suitable for use throughout the discovery - development process.

Leuprolide is a gonadotropin-releasing hormone agonist (GnRH agonist) with the amino acid sequence p-Glu-His- Trp-Ser-Tyr-D-Leu-Leu-Arg-Pro-NHEt. This report describes a method by which a crude sample of solid phase synthesized leuprolide can be purified on a PLRP-S 100Å 10 µm column.

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Materials and Reagents

Sample Preparation

A StratoSpheres PL-Rink resin was used for the solid phase synthesis of a crude quantity of leuprolide. 1 mg/mL solutions of the crude peptide were then used for the initial screening work.

Mobile Phase Preparation:

Eluent A:	0.1% TFA in 1% ACN: 99% water
Eluent B:	0.1% TFA in 99% ACN: 1% water
Flow Rate:	1 mL/min
Detection:	UV at 220 nm

Method Development

Following an initial 20 minute scouting gradient from 0-100% B on a high performance PLRP-S 100Å 5 µm 250 x 4.6 mm column, the gradient profile was modified to 25-40% B in 20 mins at a flow rate of 1 mL/min. This gave retention for leuprolide at around 16 minutes. Figure 1 shows the separation of the leuprolide on the analytical column.

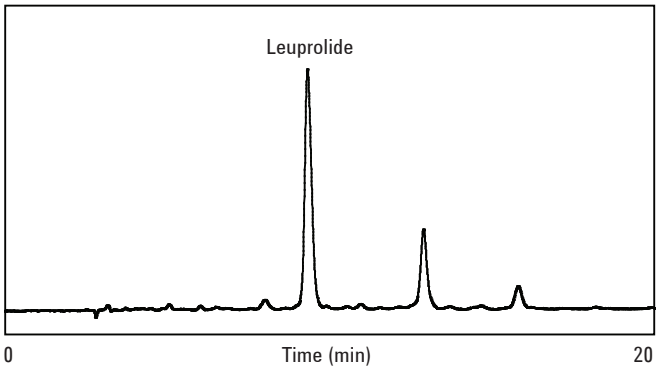


Figure 1. Separation of crude leuprolide, 20 µL of 1 mg/mL solution, on PLRP-S 100Å 5 µm, 250 x 4.6 mm ID column. Gradient: 25-40% B in 20 mins at a flow rate of 1 mL/min (360 cm/hr).

The impurity peaks elute close to the product peak and therefore it may be possible to use isocratic elution. To assess this option a plot of ACN content vs retention was produced (Figure 2). This plot indicated that PLRP-S 100Å 10 µm with isocratic elution is suitable for the purification when an acetonitrile content of between 31 and 26% is used.

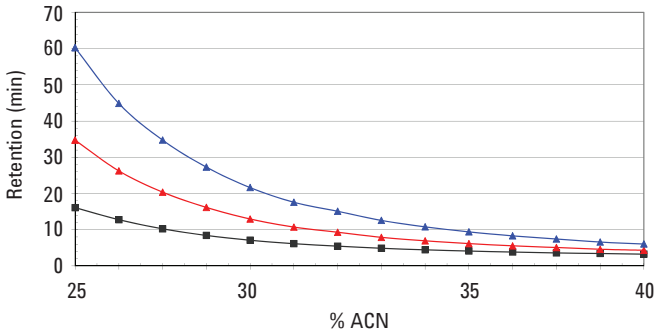


Figure 2. Plot of acetonitrile content vs retention time for leuprolide and by-products.

Loading Study

Isocratic elution conditions of 0.1% TFA in 28% ACN:72% water were chosen, therefore the next stage was a loading study using the PLRP-S 100Å 10 µm 250 x 4.6 mm analytical size column. The column dimensions required to purify a typical small scale synthesis, 30 to 50 mg, were determined to be a 1 in. Therefore, for a 30 mg purification of crude leuprolide a 1 in. Load & Lock dynamic axial compression (DAC/SAC) column was required. The linear scale up factor from a 4.6 mm ID to a 1 in. (actual ID is 27 mm) L&L column is 34 when the length is kept constant at 250 mm.

As the leuprolide is readily soluble in the eluent, concentration overload can be used. A 30 mg load in a 1 mL injection volume on the L&L column would be equivalent to a 0.9 mg load in a 30 µL injection volume (÷ 34). Figure 3 shows the analytical load (A) and prep load (B) on the 4.6 mm ID column.

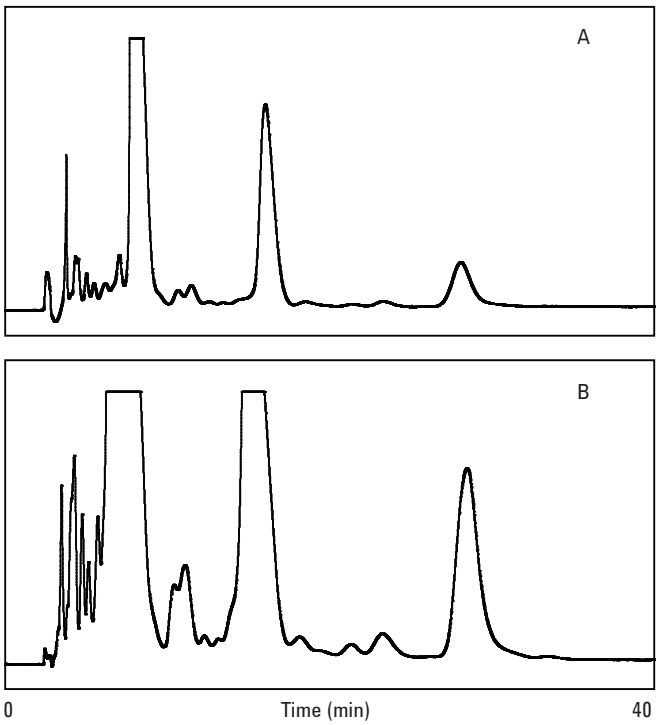


Figure 3. Crude leuprolide, on the PLRP-S 100Å 10 µm 250 x 4.6 mm ID column with a linear velocity of 360 cm/h. Isocratic separation using 0.1% TFA in 28% ACN: 78% water. A). Analytical loading of 0.2 mg (200 µL of a 1 mg/mL solution), B). Preparative loading of 0.9 mg (30 µL of a 30 mg/mL solution).

Packing of a 1 in. L&L Column

For a 250 mm x 1 in. L&L column, 40 g of dry PLRP-S 100Å 10 µm was required. This was dispersed in 175 mL of the packing solvent, 80:20 v/v acetonitrile/water, to give a final slurry concentration of approximately 0.23 g dry PLRP-S per mL of packing solvent.

After mixing on a bottle roller for 30 minutes, the slurry was poured into the assembled column and the piston pressure set to 650 psi (NB: hydraulic pressure set to 260 psi as the compression ratio for a 1 in. L&L is 1:2.5). After packing, the column plunger was locked in the compressed position so that the column could be operated in static axial compression (SAC) mode, the optimum for PLRP-S.

For this application, the efficiency achieved on the 1 in. L&L column, 50,000 ppm and symmetry 1.37, was equivalent to the analytical column, 41,000 ppm and symmetry 1.19. Therefore, when using the same linear velocity, a comparable separation would be expected.

Results

As sufficient resolution for the purification was achieved in the loading study, a 30 mg load in a 1 mL injection volume can be made onto the 250 mm x 1 in. L&L column to purify the peptides via concentration overload. Figure 4 shows the concentration overload chromatogram.

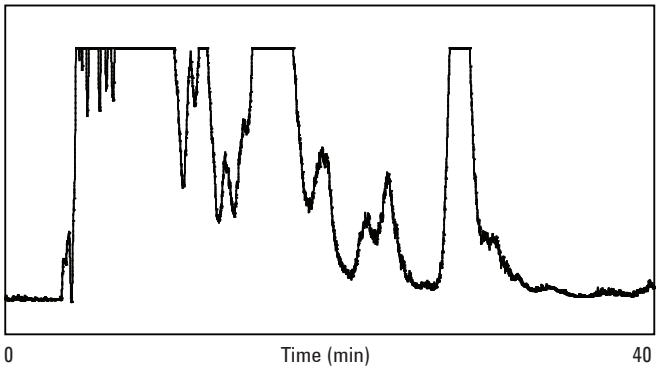


Figure 4. Crude leuprolide separation of 30 mg on-column load. PLRP-S 100Å 10 µm 250 x 27 mm ID L&L column. Isocratic separation using 0.1% TFA in 28% ACN:72% water at a linear velocity 360 cm/hr.

Fractions were collected on a time basis, 30 second, across the peaks and the fractions analysed using the gradient method and the PLRP-S 100Å 5 µm, 250 x 4.6 mm ID column. Figure 5 shows the analysis of three of the fractions, 7 with the early eluting by-products, 11 which is the leuprolide and 16 which contains the later eluting by-product.

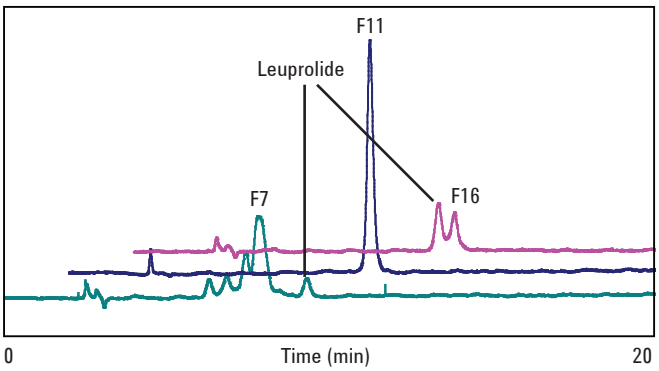
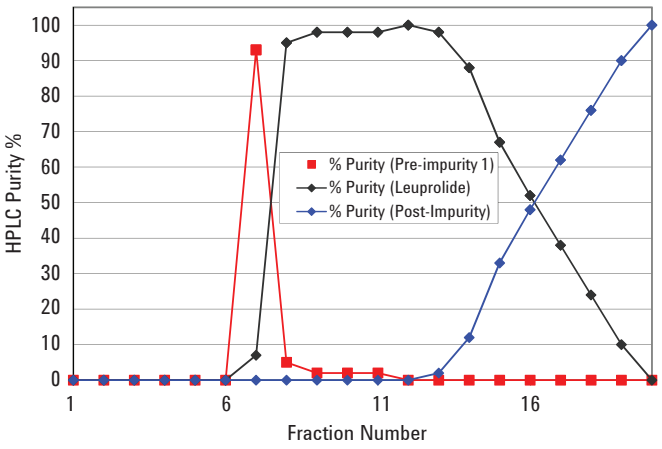


Figure 5. Leuprolide fraction analysis, PLRP-S 100Å 5 µm, 250 x 4.6 mm ID.



The quantitation of the individual fractions over the elution of the leuprolide is shown in Figure 6. The purification from the early eluting by-product is relatively efficient, but at this loading the leuprolide peak tails into the later eluting byproduct peak which is itself relatively broad and spread over a number of fractions.

Combining fractions for purity and yield analysis showed that using the concentration overload regime a purity of 98.3% was achieved with a yield of 46.8% and of 96.1% with a yield of 82.4%. The purity of the crude peptide was 62%.

Conclusion

Initial screening for a peptide purification can be done using a small particle version of the media which has the same selectivity.

Analytical sized columns packed with the purification material can be used for the loading study.

Leuprolide can be purified using concentration overload with isocratic elution.

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