METHODS IN MOLECULAR BIOLOGY™

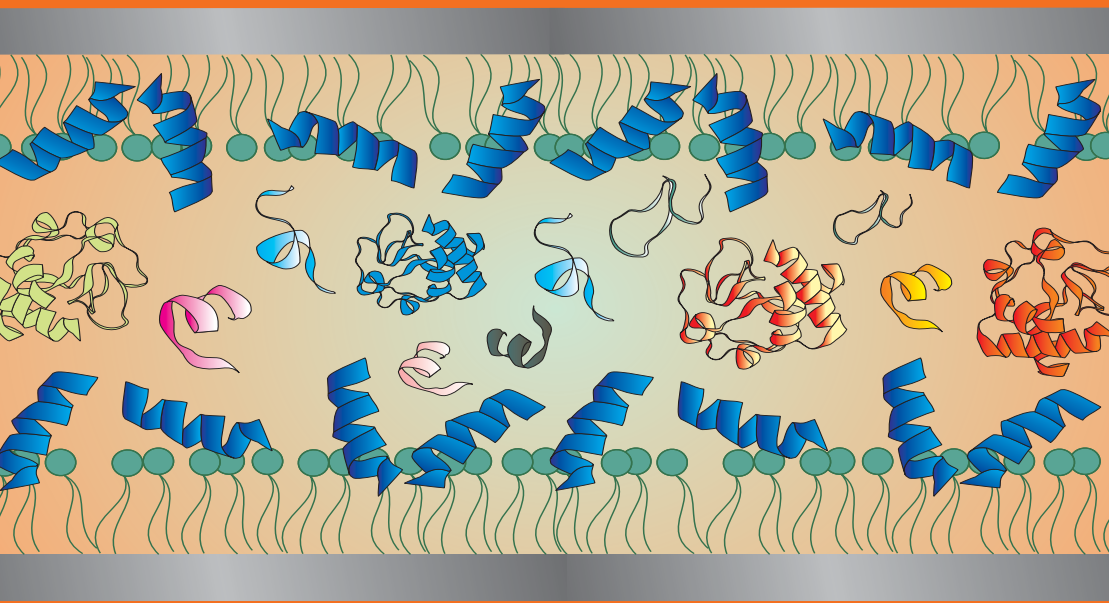
Volume 251

HPLC of Peptides and Proteins

Methods and Protocols

Edited by

Marie-Isabel Aguilar



HUMANA PRESS

14

Prep/Semiprep Separations of Peptides

Denis B. Scanlon and James Finlayson

1. Introduction

Peptide synthesis has undergone a major transformation in the last three decades, building on the solid-phase synthesis methodology of Bruce Merrifield first published in 1963 ***(1)***. During the 1970s, the first automation of peptide synthesis was undertaken using Boc chemistry. In the 1980s, improvements were made in the Boc chemistry automated process and, consequently, the synthesis of more-difficult sequences, as well as longer polypeptides became possible ***(2)***. Solid-phase Fmoc synthesis was developed in the early 1980s ***(3,4)*** and was also applied to automated systems ***(5)***. The 1990s saw improvements in both Boc and Fmoc chemistry together with novel modes of activation of the amino acids in both chemistries ***(6,7)***. The result was faster cycle times and, hence, reduced synthesis times. The range of protecting groups and resins available today means that sophisticated syntheses utilizing a combination of Boc and Fmoc chemistry are possible ***(8)***.

Despite the improvements in chemistry, the possible permutations of the 20 amino acids in a peptide of approx 25–30 amino acids in length results in biomolecules with extraordinary differences in physical properties. Purification of a crude peptide mixture can hold great challenges. Apart from chemical failures resulting in deletions, terminations, or modifications of the desired sequence ***(9)***, other problems such as poor solubility or tertiary structure in the presence of C_{18} reversed-phase (RP) silica require a many faceted approach to the purification of the humble peptide. There is a high demand for peptides as research tools and as potential therapeutic agents. The peptide chemist, therefore, has to possess the skill to synthesize and purify from milligram quantities to multigram quantities.

From: *Methods in Molecular Biology, vol. 251, HPLC of Peptides and Proteins: Methods and Protocols*
Edited by: M.-I. Aguilar © Humana Press Inc., Totowa, NJ

This chapter will deal with preparative and semipreparative approaches to peptide purification and will describe purifications in the range of 50–2000 mg of crude material. RP is the most frequent mode of purification and we will present buffer variations and discuss changes in the RP support. We will describe the integration of RP purification into synthesis strategies where isolation and purification of intermediates are integral to the overall success of the synthetic strategy. Whereas detailed mass spectrometric methods will not be discussed in this chapter, the importance of mass spectral data will be stressed in the analysis of crude peptide mixtures, the choice of a high-performance liquid chromatography (HPLC) purification strategy and as a final QC tool for purified peptides.

The purification process is a multistep process and the aim of this chapter will be to describe each step in a simple and logical manner (*see* **Note 1**). Some peptides can be extremely difficult to purify for diverse reasons. It is not the aim of this chapter to make the reader an instant expert. Rather, it is to provide a starting point for developing more complex purification strategies.

2. Materials

2.1. Instruments

2.1.1. Preparative System

1. Waters Delta Prep 3000 Quaternary Pumping System.
2. Waters Model 486 LC Spectrophotometer.
3. Waters 745 Data Module.
4. Isco Foxy 200 fraction collector.

2.1.2. Analytical System

1. Waters 600 Quaternary Pumping System.
2. Waters Model 486 LC Spectrophotometer.
3. Waters 717plus Autosampler.
4. Waters Millenium Software.

2.2. Columns

2.2.1. Preparative System

1. Amicon 75 × 50 mm stainless-steel column packed with Merck Lichrospher 100 RP-18 (12 μm).
2. Waters Prepak Cartridge, PrepNova-Pak HR C_{18}, 6 μm, 40 × 100 mm.
3. Waters Prepak Cartridge, PrepNova-Pak HR C_{18}, 6 μm, 25 × 100 mm.
4. Phenomonex Aqua column, 5 μm, C_{18}, 20 × 60 mm.

2.2.2. Analytical System

1. Merck Lichrocart 125-4 cartridge, C_{18}, 5 µm, 4.6 × 125 mm (crude QC, fraction analysis).
2. Merck Superspher 250-4 cartridge, C_{18}, 5 µm, 4.6 × 250 mm (final QC).

2.3. Reagents

The following is not an exhaustive list of suppliers, but care must be taken in choice of supplier for each reagent. All reagents should be of the highest possible quality.

1. Acetonitrile, Merck, HiPerSolv for HPLC.
2. TFA, Auspep, redistilled.
3. TEA, Auspep, redistilled.
4. Phosphoric acid, BDH Analar (85% aqueous solution).
5. Ammonium acetate, Sigma, SigmaUltra grade.
6. Acetic acid, Glacial, Aldrich >99%.

2.4. Buffers

In all cases Buffer A is the major aqueous component and Buffer B contains a majority of organic modifier.

1. TFA buffer, pH 2.0 ***(10)***: Buffer A: 0.1% aqueous TFA. Buffer B: 90% acetonitrile/water (containing 0.1% TFA).
2. 100 m*M* TEA.Ac, pH 5.5: Buffer A: 100 m*M* TEA titrated to pH 5.5 with glacial acetic. Buffer B: 50% acetonitrile/water containing 100 m*M* TEA titrated to pH 5.5 with glacial acid.
3. 100 m*M* ammonium acetate, pH 5.5: Buffer A: 100 m*M* NH_4OAc titrated to pH 5.5 with glacial acetic acid. Buffer B: 50% acetonitrile/water containing 100 m*M* NH_4OAc titrated to pH 5.5 with glacial acid.
4. Phosphoric acid buffer, pH 2.0: Buffer A: 0.1% aqueous phosphoric acid. Buffer B: 50% acetonitrile/water (containing 0.1% phosphoric acid).
5. TEA.Phosphate buffer system ***(11)***: Buffer A: 100 m*M* TEA, pH titrated in the range 2.5–7.5 with phosphoric acid. Buffer B: 50% acetonitrile/water containing 100 m*M* TEA, pH titrated in the range pH 2.5–7.5 with phosphoric acid.

3. Methods

Figure 1 defines the process. It starts from crude lyophilized peptide from which detailed mass spectral and analytical RP-HPLC data are obtained. Preparative conditions to achieve the separation are then defined. The final part of the process, which is equally as important as all other steps, is final QC of the lyophilized peptide. All the steps in **Fig. 1** will be discussed in turn.

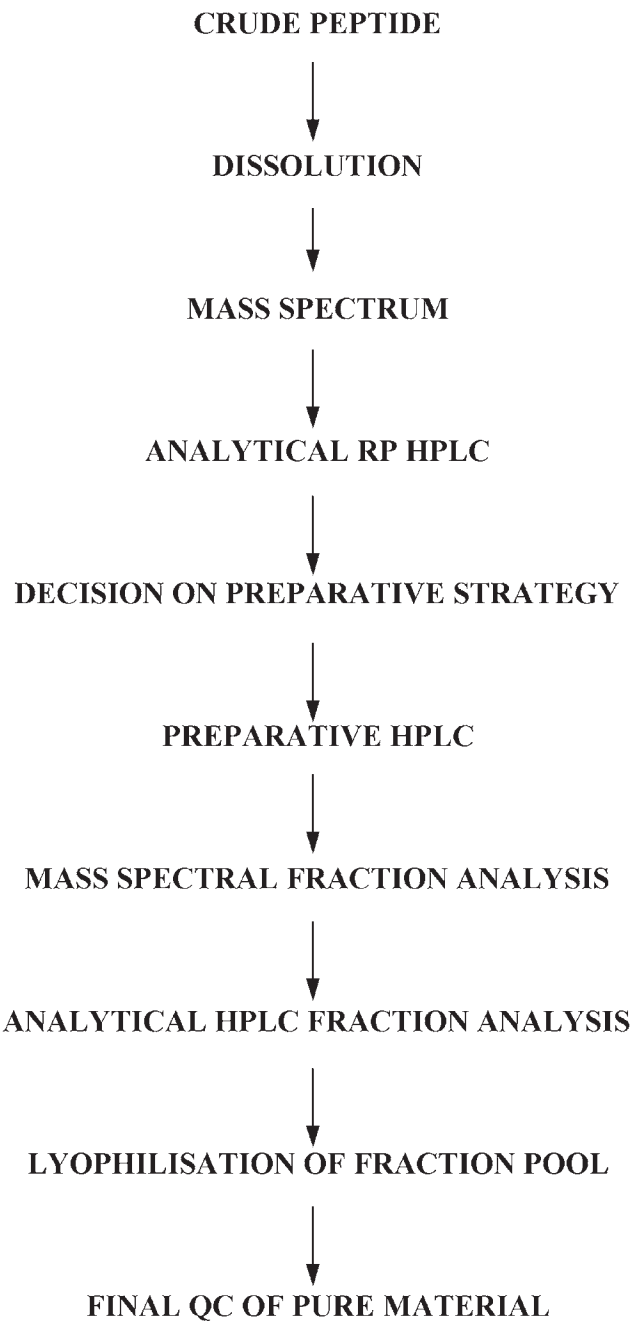


Fig. 1. Flow diagram or the process of peptide purification. Each step in the process will be discussed in turn.

3.1. Crude Sample Preparation

3.1.1. Lyophilization

For the sake of this chapter, it is assumed that the crude peptide is a lyophilized powder and the product of either a Boc or Fmoc solid-phase peptide synthesis. The sample size of crude peptide can range from 50 mg to several grams. Lyophilization removes all volatile organic scavenger from the peptide so that the crude product should be a mixture of the desired sequence together with small amounts of terminated sequences, deletion sequences, or chemically modified sequences from side-reactions during either the synthesis or the cleavage.

3.1.2. Dissolution

Before obtaining analytical data or carrying out any preparative work, the peptide must be dissolved in an aqueous buffer. Dissolution can present special problems. For ease of pumping onto a preparative column, the volume must be kept to a minimum (e.g., 50 to 200 mL). A sample concentration of around 5 mg/mL is desirable (but not crucial). This is more concentrated than the solution required to obtain analytical data (usually 0.5 mg/mL). A simple aqueous buffer is best (e.g., CH_3CN/water) as this will allow the sample to be pumped onto the preparative column without problems such as producing substantial column back pressure (causing the pumps to shut down). Simple is not always possible, however, and **Note 2** lists many options for dissolution of difficultly soluble peptides.

3.1.3. Filtering

Samples must be filtered before pumping onto any column. Failure to do this will block the porous frit end fittings on all analytical and preparative columns. Once blocked, these frits are very difficult to clean. There are many filtration membranes available commercially, but the simplest filtration device is Whatman No. 4 filter paper which acts as a 1-µm filter. Preclarification of the crude peptide solution by centrifugation is useful in cases of poor solubility. A 0.45-µ*M* membrane filter can also be used after the filter paper step but in our experience is seldom necessary.

3.2. Analytical Data

A critical step in the purification process is to obtain detailed analytical data on the crude peptide. The mass spectrum gives vital information regarding the nature of crude peptide; first, whether the crude sample has the correct molecular weight and second, the nature of the impurities in the crude peptide mixture. **Table 1** lists many of the possible deletions or modifications that can occur

Table 1
Amino Acid Modifications

Mass change	Deletion	Mass change	Modification
–186	Trp	+16	Met[O]
–163	Tyr	+22	Na+
–156	Arg	+28	Formyl
–147	Phe	+44	CO_3(fromTrp(Boc))
–137	His	+56	tBu
–131	Met	+71	Acm
–129	Glu	+80	Phosphorylation
–128	Gln,Lys	+80	Sulfation(of Tyr)
–115	Asp	+90	Benzyl
–114	Asn	+100	Boc
–113	Leu,Ile	+104	MeBenzyl
–103	Cys	+114	TFA salt
–101	Thr	+120	MeOBenzyl
–99	Val	+165	DDE
–97	Pro	+222	Fmoc
–87	Ser	+242	Trt
–79	desPhospho	+252	Pbf
–71	Ala,desAcm	+266	Pmc
–57	Gly		
–18	Dehydration(–H_2O),Aspartimide		
–17	Pyroglutamic acid from Gln		
–2	Disulphide bond formation		

[a]Possible modifications or deletions present in crude synthetic peptides. The list is far from comprehensive but attempts to document the commonly observed amino acid deletion masses or protecting group modification masses.

in peptide synthesis. The nature of deletions and modifications will influence the RP-HPLC conditions chosen for the preparative separation (*see* **Note 3**).

If the mass spectrum shows the correct molecular ion, then a C_{18} RP-HPLC trace is run, usually in TFA buffer system with an acetonitrile gradient. A typical gradient will run from 0 to 60% acetonitrile over 30 min. If the peak shape is broad or the resolution is poor then a second analytical trace in TEA.phosphate or ammonium acetate buffer (*see* the buffer list in **Subheading 2.4., item 5**) is run to optimize the separation. Peptides will generally elute between 10 and 25 min under these running conditions. If the peptide is extremely hydrophobic and is retained longer than 25 min then the retention time of the peptide on a C_4 or Cyano(CN) RP-HPLC column (less hydrophobic support)

should be investigated. Superior preparative results will be obtained if the peptide elutes in the 10–25-min range analytically. Peptides eluting outside this range generally give poor recoveries from the purification process (*see* **Note 4**).

Hydrophilic peptides eluting before 10 min should be examined analytically with shallow gradients from 0 to 15% acetonitrile over 30 min. Highly end-capped C_{18} RP-HPLC columns (e.g., *see* **Subheading 3.6.4.**) retain hydrophilic peptides in cases where standard columns do not.

3.3. Prep System Setup

3.3.1. Choosing the RP-HPLC Column and Buffer

The choice of the preparative column and buffer is a direct scale-up from the results obtained in the analytical data. If a nonvolatile buffer is selected for the preparative separation, then desalting the purified peptide in volatile TFA buffer, or in some cases, acetic acid buffer (*see* **Note 5**) will be required. The dimensions of the preparative column depends on the quantity of peptide to be purified and the complexity of the crude product. A semipreparative column with the dimensions 25 × 100 mm (e.g., Waters 25 × 100-mm NovaPrepPak cartridge detailed in **Subheading 2.2.1.**) has the capacity to handle 50–200 mg of crude peptide. Larger quantities of peptide (200 mg to multigrams) are best purified on a preparative column with the dimensions 40 × 100 mm or larger (e.g., the NovaPrep-Pak detailed in **Subheading 2.2.1.**). The column capacity increases proportionally with the square of the column radius and in our experience a column with dimensions 75 × 50 mm (short and fat) can purify up to 10 g of crude peptide in a single preparative run (*see* **Note 4**). If a purification is complex, that is the crude chromatogram is complex and the integrated percentage of peptide in the crude trace is low, then results will improve at the lower levels of the theoretical capacity range of the preparative column (i.e., smaller batch amounts purified on a larger dimension column).

3.3.2. Defining the Gradient

A gradient must be set up on the software of a quaternary pumping sytem (with inlet lines A, B, C, D). Starting conditions are usually in the range 0–10% Buffer A and the gradient develops with a slow linear increase in Buffer B concentration (e.g., 0.3–0.5% Buffer B/min). Generally, a linear gradient from 0 to 50% acetonitrile is a good starting point for relatively soluble peptides, and, a gradient from 20 to 80% acetonitrile is appropriate for more hydrophobic peptides (*see* **Section 3.4.2.**). If more detail is known about the elution properties of the peptide compared to the impurities (*see* **Note 3**) then a complex gradient (e.g., nonlinear or with fast step-ups to particular Buffer B concentrations followed by a slow linear gradient) can be designed. The sample is introduced isocratically through line D prior to the commencement of the gradient.

3.3.3. Detection

The product of the gradient elution from the column will flow through a detector and the detection wavelength will be set according to the composition of the peptide. The absorbance of the peptide bond can be monitored at 214–220 nm in analytical mode, but in preparative mode the wavelength should be "tuned out" to make the detection less sensitive. The product should therefore be monitored at 225–232 nm depending on how much sample has been introduced onto the column (*see* **Note 6**).

3.3.4. Fraction Collection

The next set-up task is the programming of the fraction collector. The volume of the fractions collected will depend on the flow rate used, the size of the fraction tubes and the difficulty of the separation to be achieved. These decisions are better made before the introduction of the sample onto the column.

Typically, 0.5-min fractions are a good starting point, but the final volume depends on the difficulty of the separation to be achieved. The fraction collection can be run totally in time mode where fractions are collected over the whole preparative run, or, alternatively, in peak detection mode. Most fraction collectors can be electronically linked with the detector. When a peak is detected (i.e., the absorbance level becomes greater than a predetermined baseline level) the fraction collector switches into collect mode. When the product has eluted the absorbance will fall and the fraction collector will switch off at the same predetermined baseline absorbance level (*see* **Note 7**).

3.3.5. Equilibration of the Column

The final set-up task is equilibration of the system. Some peptides or non-peptidic material can be strongly retained on the column and failure to flush out this material may result in broad "ghost" peaks in subsequent separations. Prior to commencing the preparative run, therefore, a column wash with 100% Buffer B is performed (three column volumes) followed by re-equilibration to Buffer A conditions (five column volumes) (*see* **Note 7**). The sample introduction line D should be washed with water.

3.4. The Prep Run

3.4.1. Pumping On

Because of the volumes involved in the dissolution of peptides in the prep or semi-prep mode, the preparative system must be set up so that the sample can be pumped onto the column. Introduction of the sample through a rheodyne injector is not practical. A quaternary pumping system is required for the

task. The filtered buffers are installed in lines A (aqueous) and B (organic) and the introduction of the sample is through line D.

Using the isocratic control section of the software, the peptide solution is introduced on to the column through line D as a mixture with Buffer A. The composition of this mixture will depend on the amount of organic modifier required to solubilize the peptide and the "stickiness" of the peptide on a RP column. Peptides that elute early from an analytical run (20% Buffer B or less) would need to be pumped on at 80% Buffer A/20% line D to ensure the peptide is retained on the column rather than crashing through before the gradient is commenced (*see* **Note 7**). Peptides that are better retained can be pumped on at 50% Buffer A/50%/lined D (*see* **Note 9**). Once the peptide solution is on the column, line D is washed (*see* **Note 10**) to ensure all the sample has been loaded, and, to clean line D ready for the next preparative run.

3.4.2. Starting the Gradient

If a strongly absorbing agent has been used to aid in the dissolution of the peptide (acetic acid, dimethyl sulfoxide [DMSO], or dimethyl formamide [DMF] for instance), the detector will be off-scale during the pumping on stage. After all the peptide solution has been introduced onto the column, Buffer A is pumped isocratically until the absorbance returns to baseline. Once the absorbance has returned to baseline, the gradient is started and the fraction collector set to peak detection mode. Until a peak is detected, the fraction collector diverts the flow to a waste bottle (*see* **Note 7**). Where throughput is desired, however, a robust reproducible method is required which is applicable in almost every case.

3.4.3. Monitoring the Preparative Run/Fraction Collection

Preparative systems with computer-controlled software are capable of continuous monitoring of the preparative trace and have options for running with the product peak spanning full-scale deflection on the monitor or rescaling to view small impurities. Fraction collection parameters are all controlled by the software in such systems and give the chromatographer total flexibility in monitoring the preparative trace (*see* **Note 8**).

Many older preparative systems may only have a chart recorder or data module with the facility to nominate the range of absorbance that will be plotted onto a paper chart. The absorbances suitable for preparative scale chromatography will only be those in the upper end of the range, namely either 0.5, 1.0, or 2.0 absorbance units full scale (Aufs). It is also essential that the fraction collector and the detector be interconnected such that whenever a new

fraction is collected a relay is closed that produces an absorbance spike, and, therefore, a mark on the preparative trace. In this manner, the exact position of each fraction can, therefore, be determined on the preparative trace.

3.4.4. Fraction Analysis

Running full analytical RP-HPLC traces for each fraction collected is an incredibly time-consuming process and analytical HPLC time in most laboratories is usually at a premium. The preferred method to initially screen fractions is electrospray mass spectrometry. A complete set of fractions from a preparative run can be screened in less time than an analytical RP-HPLC run of a single fraction. Moreover, the quality of the raw mass spectral data can be an excellent guide to the purity of the fraction being analyzed. One must be cautious, however, as good mass spectral data does not guarantee the RP-HPLC trace will be to the required purity specification. In the case of closely eluting peaks, qualitative mass spectrometry can be used as a guide to locate the peak coresponding to the desired peptide. Based on the mass spectral data, pools of fractions or individual tubes can then be run analytically on RP-HPLC. The tubes exhibiting the required purity by analytical HPLC are pooled and lyophilized (*see* **Note 11**).

3.5. Final QC

After lyophilization, the integrity of the dry-powder product must be confirmed. There are times when a peptide is sensitive to a range of handling conditions and during the lyophilization process the purity by RP-HPLC can decrease by a small amount (or in rare cases substantially) from that assessed in a fraction pool (*see* **Note 12**). To guarantee that the final product is of the desired purity, the pure, dried peptide must be subjected to analytical RP-HPLC, mass spectrometry, and in some cases, amino acid analysis.

3.6. Examples

Analytical and preparative traces obtained in the purification of the examples below will illustrate the points made in the text of the chapter.

*3.6.1. Substance P **(12)***

Figure 2 illustrates a large scale purification from a synthesis of Substance P on a scale of 2 g. Because of the quantitiy of peptide, the Amicon 75 × 50 mm preparative column was used to enable the loading of multigram quantities each preparative run. The analytical data for Substance P (*see* **Fig. 2A**) shows that it runs with good resolution in TFA buffer and the two later eluting impurities

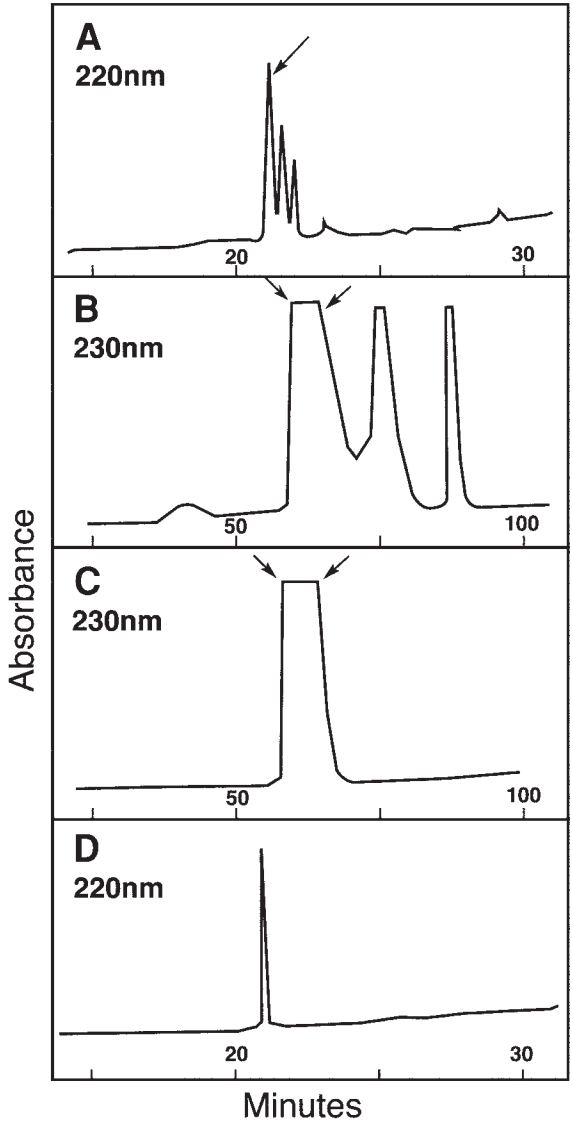


Fig. 2. Substance P: **(A)** Crude analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over 30 min. **(B)** Prep trace, 2 g sample: TFA buffer, 30 mL/min, 0–50%B over 100 min. **(C)** Prep desalt, 360 mg sample: Acetic acid buffer, 30 mL/min, 0–50%B over 100 min. **(D)** Pure analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over 30 min.

are well resolved. The mass spectral data for the crude peptide showed the correct ion for Substance P at m/e 1347 plus peptide impurities with Arg, Lys, and Arg+Lys deletions (data not shown). Owing to the hydrophilic nature of the Arg and Lys residues, these deletion peptides are more retained on C_{18} RP-HPLC and hence elute later in the chromatogram (*see* **Note 2**). The Substance P peak is well resolved from the later eluting impurities. A single preparative run in TFA buffer (*see* **Fig. 2B**) was shown by mass spectrometry to remove the Arg and Lys deletion peptides. The pooled fractions were assessed by analytical RP-HPLC and yielded 360 mg > 80% pure A final preparative run on NovaPrep-Pak 40 × 100 mm column, with acetic acid instead of TFA as the buffer counter-ion (*see* **Fig. 2C**), improved the purity to > 95% and exchanged the peptide counter-ion from trifluoroacetate to acetate (*see* **Note 5**). The final yield of pure Substance P (*see* **Fig. 2D**) was 140 mg > 95% pure.

3.6.2. Conotoxin GVIA ***(13)***

Conotoxin GVI is a polypeptide toxin from the sea snail *Conus geographus* and contains three disulfide bonds. The peptide was synthesised by Fmoc solid-phase peptide synthesis, cleaved and air oxidized in 200 m*M* Tris-HCl, pH 8.3 to form the three disulfide bonds. The crude peptide (650 mg), which by analytical RP-HPLC appeared as a peak sitting on an oxidative hump (*see* **Fig. 3A**), was initially purified in TFA buffer on a PrepNova-Pak, 6 µ*M*, 40 × 100 mm column to remove the majority of impurities and bring the peptide up to 80% purity. Repeated preparative chromatography in this buffer system, from experience in our laboratory, did not improve the purity further. Analytically 25 m*M* ammonium acetate buffer, titrated to pH 5.0 with TEA, was found to shift the oxidative hump away from the main product peak. In PITC amino acid analysis, it has been found that peak shape and resolution are enhanced in ammonium acetate buffer if the pH is titrated to below pH 5.6 with TEA. This proved to be the case for Conotoxin GVIA. When passed through the same column with ammonium acetate buffer, the oxidative hump was shifted to a resolvable later eluting peak (*see* **Fig. 3B**). On lyophilization, ammonium acetate buffer is partially, but not completely, volatile. To complete the purification (*see* **Fig. 3C**), the peptide was finally desalted on the PrepNova-Pak, 6 µ*M*, 25 × 100 mm semiprep column in 0.25% acetic acid buffer to yield 25 mg of Conotoxin GVIA at a purity of >95% (*see* **Fig. 3D**).

3.6.3. Conotoxin MII ***(14)***

Purification can be incorporated as part of a multistep synthesis strategy. This concept was applied to the synthesis and purification of Conotoxin MII, which

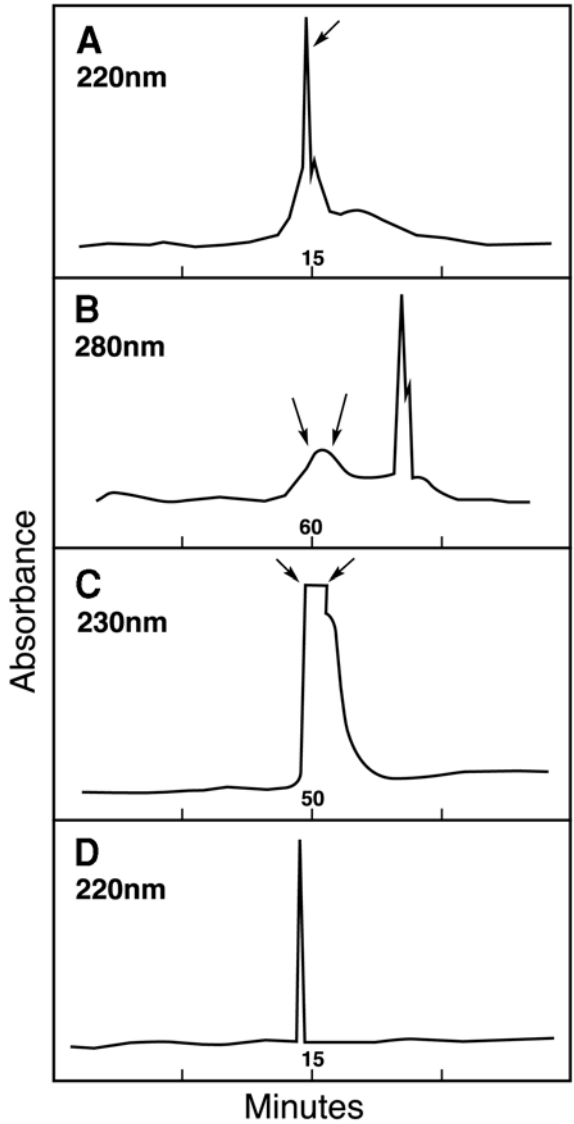


Fig. 3. Conotoxin GVIA: **(A)** Crude analytical trace (air oxidised peptide): TFA buffer, 1.5 mL/min, 0–60%B over 30 min. **(B)** Prep trace, 650 mg sample: TFA buffer, 30 mL/min, 0–15%B over 35 min, 15%B isocratic 35–45 min, 15–45%B 45–135 min, 280 nm detection. **(C)** Prep trace: 25 m*M* ammonium acetate buffer, same gradient as in (B). **(D)** Pure analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over 30 min.

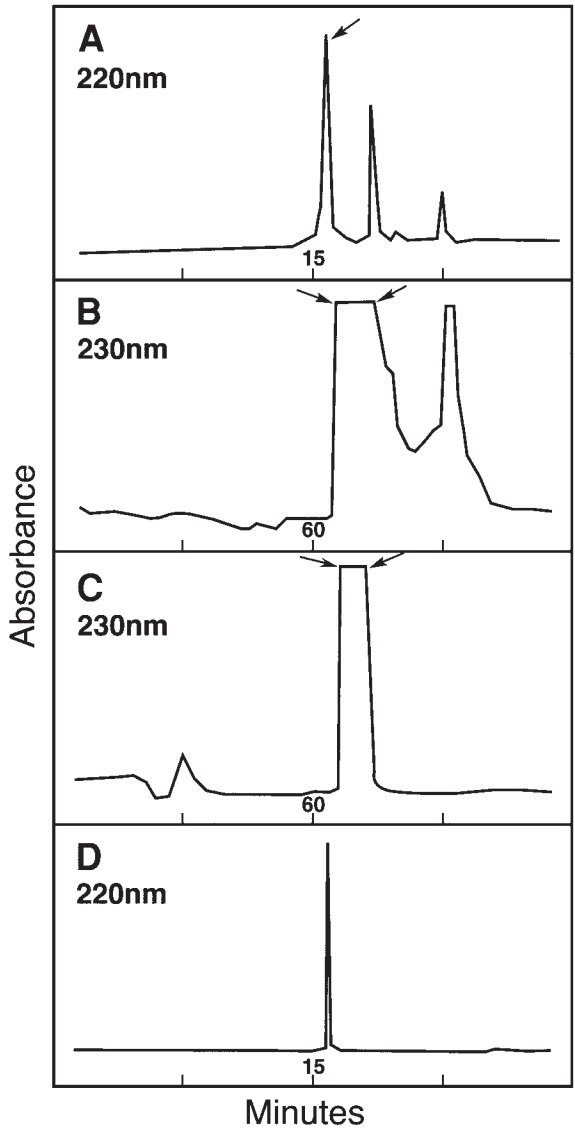


Fig. 4. Conotoxin MII: **(A)** Crude analytical trace (1 disulfide bond, 2× Cys(Acm)): TFA buffer, 1.5 mL/min, 0–60%B over 30 min. **(B)** Prep trace, 1.8 g sample (1 disulfide bond, 2× Cys(Acm)): TFA buffer, 30 mL/min, 0–50% B over 100 min. **(C)** Prep trace (2× disulfide bonds), 180 mg sample: 25 m*M* ammonium acetate buffer, 30 mL/min, 0–12%B over 30 min, 12%B isocratic 30–40 min, 12–40%B 40–100 min. **(D)** Pure analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over 30 min.

is a complex peptide from the venom of the sea snail *Conus magus* and has two disulfide bonds. The disulfide bonds were formed sequentially and selectively by the removal of two pairs of orthogonal Cys protecting groups, namely two Acm groups and two Trityl groups. The Trityl groups were removed during the postsynthesis cleavage of the peptide and the first disulfide bond formed by air oxidation (*see* **Fig. 4A**). The Acm-protected peptide (1.8 g) was purified at this point on a PrepNova-Pak, 6 µ*M*, 40 × 100 mm column in a volatile TFA buffer system to yield 180 mg of monodisulfide-Acm-protected peptide (*see* **Fig. 4B**). This process removed any impurities or excess reagent which could potentially interfere with the oxidative formation of the second disulfide bond.

The second disulfide bond was formed by oxidative removal of the Acm protecting groups. The product of this process was again purified on the PrepNova-Pak, 6 µ*M*, 40 × 100 mm column but with 25 m*M* ammonium acetate buffer pH 5.0, which removed the oxidative impurities in the same manner as that described for Conotoxin GVIA (*see* **Fig. 4C**). To complete the purification, the peptide was finally desalted on a PrepNova-Pak, 6 µ*M*, 20 × 100 mm semiprep column in 0.25% acetic acid buffer to yield 75 mg of Conotoxin MII >95% pure (*see* **Fig. 4D**).

3.6.4. c(RSRNR)

Cyclic(RSRNR) is an extremely hydrophilic cyclic pentapeptide which is poorly retained on C_{18} RP silica HPLC columns. **Figure 5A** shows an analytical trace of c(RSRNR). The early eluting nature of the peptide (9 min) made resolution of the minor back impurity (10 min) extremely difficult. The standard NovaPrep-Pak semiprep column could not retain either the cyclic peptide or the later eluting impurity. The Phenomonex Aqua semiprep column (*see* **Subheading 2.2.1.** for details), however, retained both the product and the impurity on the column for more than 25 min and effected resolution of the two components (*see* **Fig. 5B**). The loading of crude peptide was 60 mg and the yield of the cyclic pentapeptide was 15 mg > 90% pure (*see* **Fig. 5C**).

4. Notes

1. Preparative systems require a "hands-on" approach to ensure that valuable sample is not lost because of equipment malfunction. Computer driven automated systems are available, however, but will not be described as the software and hardware complexities are beyond the scope of this chapter.
2. The first buffer to try when dissolving a crude peptide is 30% CH_3CN water. If this is unsuccessful, then the following is a short list of buffers that may be useful for dissolution of insoluble peptides. As a general rule, these buffers are added to

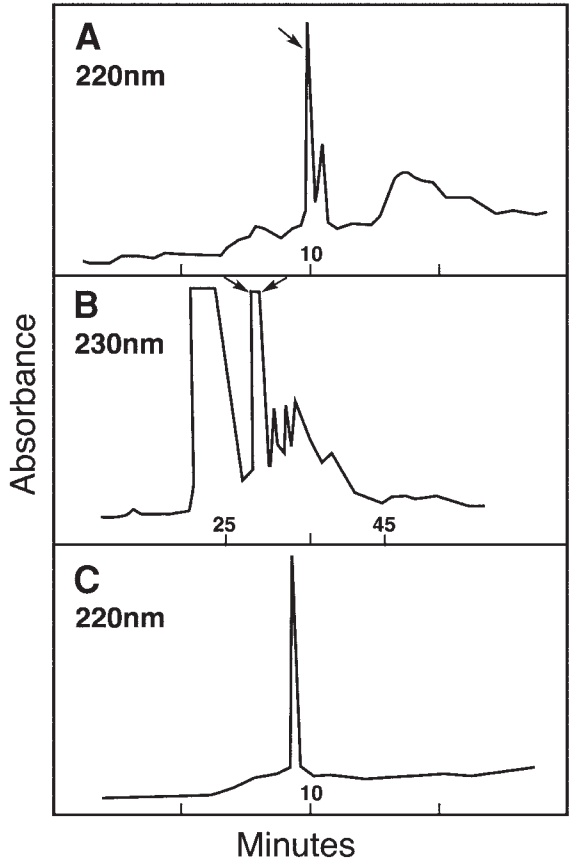


Fig. 5. c(RSRNR): **(A)** Crude analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over 30 min. **(B)** Prep trace, 60 mg sample: Phenomonex Aqua semiprep column, 0–25%B over 50 min. **(C)** Pure analytical trace: TFA buffer, 1.5 mL/min, 0–60%B over

the peptide in a concentrated form and then diluted out with water to the point where the peptide just starts to become cloudy.

a. 50% AcOH/25% water/25% CH_3CN.
b. Formic acid (neat).
c. 200 m*M* NH_4HCO_3 containing 20% CH_3CN, titrated with AcOH to pH 6.0.
d. 500 m*M* Na_2HPO_4 containing 20% CH_3CN, titrated with phosphoric acid to pH 6.0.
e. DMSO or DMF.

3. The mass spectrometer is an extremely sensitive tool for determining the range of impurities in a crude sample. Modifications of the peptide during the synthesis will elute at different times on RP-HPLC. The following are some rules of

thumb for predicting where the modifications detailed in **Table 1** may elute with respect to the desired peptide:

a. Hydrophobic amino acid deletions (e.g., I,V,L,F,M) will elute earlier.
b. Hydrophilic amino acid deletions (e.g., R,K,H) will elute later.
c. Met[O] is a common modifiication which will elute earlier and tBu adducts from the cleavage will elute later.
d. Dehydrations caused by aspartimide formation (e.g., in peptides containing the sequence D-G or D-S) will appear as a closely eluting back peak or shoulder. A number of alternate buffers must be tried to optimize the separation in such cases. Ion exchange is also an option as a negative charge has been lost in the dehydration.
e. Deletions of residues such as G,A,S,T,C,N,Q, which are readily identifiable by mass spectrometry can coelute with the desired peptide on RP-HPLC. A range of buffers and/or column packings should be tried to maximize the resolution of the deletion, but, in cases of major deletions, resysnthesis can be the best option.

4. To maximize yields, purification protocols should be devised so that the minimum number of passes through a preparative column produces the desired result. Care should be taken with extremely hydrophobic peptides, as the higher loading compared to the analytical system can, in some cases, cause the peptide to smear off the column at high acetonitrile concentrations or not come off the column at all.
5. In most cases, TFA buffer will be the initial buffer of choice because of the resolving power and the fact it is volatile on lyophilisation. Where nonvolatile salt buffers are used (e.g., phosphate buffers) a second desalting prep in volatile buffers (e.g., TFA or acetic acid) is necessary prior to lyophilization. Some biological assays, however, are sensitive to TFA being associated with a peptide as a counterion. In these cases TFA must be exchanged by acetic acid. This is most conveniently achieved by a final preparative step with 0.25% AcOH/water as Buffer A instead of 0.1% TFA ***(15)***.
6. If Tryptophan is contained in the peptide then 280 nm can be used as a detection wavelength as the indole moiety produces an absorbance maximum at this wavelength. Similarly Tyrosine has an absorbance maximum at 275 nm and the detection can be set at this wavelength if Tyrosine is contained in the peptide. Care must be taken in the choice of detection wavelength as an unexpected low absorbance response in the preparative run can mean that the fraction collector is not triggered and the product flows into the waste container.
7. Collect all eluent from the column! At times, peptides will unexpectedly not be retained (re-equilibration problems), or not elute in the fractions collected but in the "ramp-off" during the re-equilibration of the column. If all eluent from the column is collected then the peptide cannot be lost.
8. Do not expect the preparative trace to model the analytical trace. The preparative trace will be broad and most likely off-scale. Fraction analysis will confirm that resolution is occurring although it is not obvious from the trace.

9. In our experience, it is always better to dilute the sample when pumping on with Buffer A and to be cautious in choosing the percentage of Buffer A as diluent when pumping on.
10. It is often necessary to wash the line initially with a small volume of the solubilizing solvent to prevent peptide precipitation in the line before reaching the column before stepping back to water (or 10% acetonitrile depending on the gradient starting buffer conditions as described in **Subheading 3.4.2.**).
11. Sophisticated preparative systems are available where the column eluent passes through a flow splitter and is continuously monitored by mass spectrometry for a particular peptide molecular weight. Once the peptide molecular weight is detected, the fraction collector is triggered and the product is collected. Analytical RP-HPLC traces are then run to assess the purity of the fractions.
12. During lyophilization, the peptide is susceptible to several oxidative processes. Peptides containing Met can oxidize unexpectedly. Peptides containing Cys can dimerize. Peptides containing multiple Cys can cyclize internally or form disulfide-linked oligomers. The purity should be rechecked following lyophilization.

Acknowledgments

We thank Dr. Philip Mack and Paul Jones for constructive suggestions and careful of editing of the text. We also thank Trevor Smith for invaluable help with the diagrams.

References

1. Merrifield, R. B. (1963) Solid phase synthesis I. The synthesis of a tetrapeptide. *J. Amer. Chem. Soc.* **85,** 2149–2154.
2. Clark-Lewis, I., Aebersold, R., Ziltener, H., Schrader, J. W., Hood, L. E., and Kent, S. B. H. (1986) Automated chemical synthesis of a protein growth factor for hemopoietic cells, Interleukin-3. *Science* **231,** 134–139.
3. Brown, E., Sheppard, R. C., and Williams, B. J. (1983) Peptide synthesis. Part 5. Solid-phase synthesis of [15-Leucine] little gastrin. *J. Chem. Soc. Perkin Trans.* **I,** 1161–1167.
4. Scanlon, D. B., Eefting, M. A., Lloyd, C. J., Burgess, A. W., and Simpson, R. J. (1987) Synthesis of biologically active transforming growth factor-alpha by fluorenylmethoxycarbonyl solid phase peptide chemistry. *J. Chem. Soc. Chem. Commun.* 516–518.
5. Sheppard, R. C. (1986) Modern methods of solid-phase peptide synthesis. *Science Tools* **33,** 9–16.
6. Schnolzer, M., Alewood, P., Jones, A., Alewood, D., and Kent, S. B. H. (1992) *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. *Int. J. Peptide Protein Res.* **40,** 180–193.
7. Angeletti, R. H., Bonewald, L. F., and Fields, G. B. (1997) Six-year study of peptide synthesis. *Methods Enzymol.* **289,** 697–717.
8. Dawson, P. E. and Kent, S. B. H. (1993) Convenient total synthesis of a 4-Helix TASP molecule by chemoselective ligation. *J. Amer. Chem. Soc.* **115,** 7263–7266.

9. Smith, A., Young, J. D., Carr, S. A., Marshak, D. R., Williams, L. C., and Williams, K. R. (1992) State-of-the-art peptide synthesis: comparative characterization of a 16-mer synthesized in 31 different laboratories, in *Techniques in Protein Chemistry III* (Angeletti, R., ed.), Academic, San Diego, CA, pp. 219–229.
10. Bennet, H. P. J., Browne, C. A., and Solomon, S. (1980) The use of perfluorinated carboxylic acids in the reversed-phase HPLC or peptides. *J. Liquid Chromatogr.* **3,** 1353–1365.
11. Rivier, J. E. (1978) Use of trialkyl ammonium phosphate buffers in reverse phase HPLC for resolution and high recovery of peptides and proteins. *J. Liquid Chromatogr.* **1,** 343–366.
12. Tregear, G. W., Niall, H. D., Potts, S. E., Leeman, S. E., and Chang, M. M. (1971) Synthesis of Substance P. *Nature (Lond.) New Biol.* **232,** 86–89.
13. Olivera, B. M., McIntosh, J. M., Cruz, L. J., Luque, F. A., and Gray, W. R. (1984) Purification and sequence of a presynaptic peptide toxin from Conus geographus venom. *Biochemistry* **23,** 5087–5090.
14. Shon, K. J., Koerber, S. C., Rivier, J. E., Olivera, B. M., and McIntosh, J. M. (1997) Three-dimensional solution structure of alpha conotoxin MII, an alpha 3 beta 2 neuronal nicotinic acetylcholine receptor-targetted ligand. *Biochemistry* **36,** 15,693–15,700.
15. Gabriel, T. F. (1987) Simple, rapid method for converting a peptide from one salt form to another. *Int. J. Peptide Protein Res.* **30,** 40–43.