Purification of Synthetic Peptides by Reversed-Phase Flash Chromatography

Boris Polanuyer and Michael Ye

SUPELCO, 595 North Harrison Road, Bellefonte, PA 16823

T404124
HND

Introduction

Peptides are key to modern drug discovery. Millions of different peptides are currently produced in mg-scale for research purpose, in order to better understand the function of biological systems. Some newly discovered sequences form the basis of modern drugs and are now produced in multi-tons. The most popular example is the T-20 peptide (Fuzeon), which is the first peptide produced at such scale by a combination of solid phase and solution phase methodologies. This particular peptide sequence has the ability to dock on the surface of the HIV virus and block the virus from entering into a human blood cell, helping patient life

Introduction (contd.)

Purification is an essential step in production of synthetic peptides. Most popular approach for this task is preparative HPLC. Protected nonpolar peptides may be purified in normal-phase mode using silica sorbents. Polar or deprotected peptides requires reversed-phase or ion-exchange purification. High prices of preparative HPLC systems and preparative columns result in high prices of purified peptides.

Recently it was demonstrated that both normal-phase and reversed-phase flash chromatography may be applied for purification of nonpolar natural peptides – cyanobacteria' microcystins (1, 2).

The aim of this study was to evaluate recently developed flash purification system and cartridges as a tool for synthetic peptide purification.

HPLC Based Method Development

Typical approach for normal-phase flash chromatography method development is thin-layer chromatography (TLC). This approach has a lot of advantages – simple equipment, inexpensive TLC plates. Disadvantages include – it's not possible to predict gradient separation conditions for flash purification using isocratic TLC conditions. Moreover, typically reversed-phase TLC plates are not ideal for peptide separation.

Therefore reversed-phase HPLC was applied for method development in current study. Gradient HPLC requires more complex and expensive equipment, however it allows to predict separation conditions in a few runs.

Linear gradient conditions predicted by HPLC may be easily transferred into optimal step-gradient conditions for flash purification. The same HPLC system, column and conditions, as used for method development may be applied for analysis of purified product.

Experimental
Materials

Synthetic decapeptide Val-Gln-Ala-Ala-Asp-Tyr-Ile-Asn-Gly was obtained from Sigma-Genosys.

In accordance with Expasy ProtParam analysis (3) this peptide provides the following features

Molecular weight: 1063.1

Theoretical pI: 3.80 Acidic

Total number of negatively charged residues (Asp + Glu): 1

Total number of positively charged residues (Arg + Lys): 0

Extinction coefficients:Extinction coefficients at 280 nm M^{-1} cm^{-1} 1280

Aliphatic index: 127.00 Hydrophobic, strong retention on C18

All other chemicals were obtained from Sigma-Aldrich and used without further purification

Flash System

Supelco VersaFlash™ flash chromatography apparatus and Supelco VersaPak™ cartridges 75 x 40 mm I.D., packed with C18 sorbent was used for peptide purification.

Step gradient was generated using SciLog Accu pump with FMI valveless piston pump head.

Premixed acetonitrile/water solvents were used.

Selection of HPLC Conditions

Typical reversed-phase protein separation conditions are reversed-phase (C4, C8, or C18) column and linear gradient of acetonitrile/water with addition of TFA.

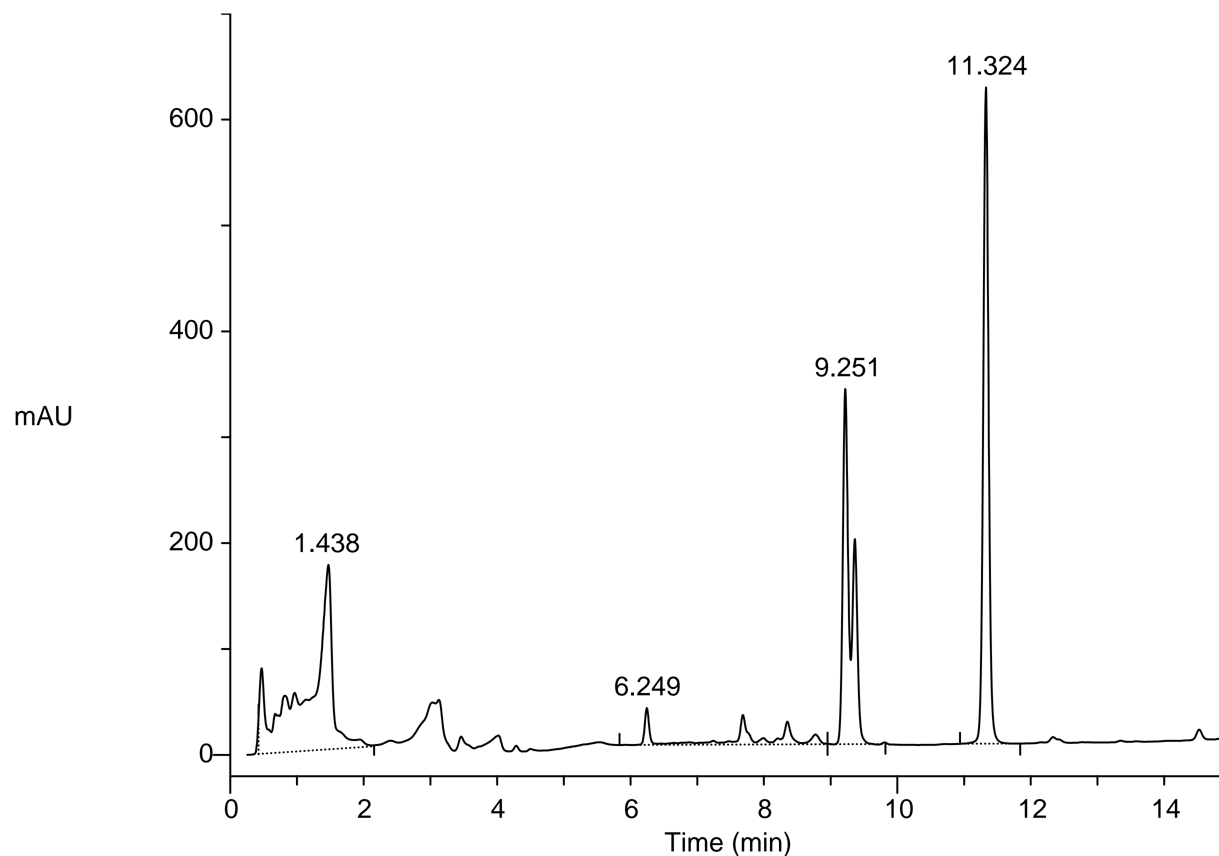
For specific purposes of this study, the C18 column is preferable because surface chemistry in reversed-phase cartridges and C18 columns is the same. Due to acidic nature of purified peptide, acidification of mobile phases with TFA is not necessary.

Therefore HPLC separation was carried out using C18 column (Supelco, Discovery® C18, 50 mm x 2.1 mm I.D. packed with 5 µm sorbent) using linear gradient of acetonitrile/water from 0 to 100% acetonitrile.

Due to presence of tyrosine residue in purified peptide, UV monitoring at 254 nm was used.

Selection of HPLC Conditions

Figure 1. HPLC of Crude Peptide



Selection of Flash Chromatography Conditions

VersaFlash system with 75 x 40 mm I.D. VersaPak cartridge, packed with spherical silica (C18 content 26%) was used. Cartridge was pre-wetted in step gradient of acetonitrile/water (20%, 40%, 100% v/v, 100 mL of each solvent) and washed with water. Peptide samples (10 mg/mL, 2 mL) were injected using stop-flow injector.

Based on HPLC results (see Figure 1) – the following step gradient conditions were used:

1. 20% acetonitrile – 200 mL
2. 60% acetonitrile – 200 mL
3. 80% acetonitrile – 200 mL
4. 100% acetonitrile – 200 mL

Flash Chromatography

100 mL fractions were collected manually and concentrated using rotor evaporator. Concentrated fractions were analyzed by HPLC. The compound of interest was found in 80% acetonitrile fraction. 95%+ purity peptide was obtained with 90% yield. Most of the impurities were concentrated in 60% acetonitrile, 100% acetonitrile fractions contained no detectable components.

Preliminary experiments demonstrate that compound of interest elutes in 80% acetonitrile. Therefore flash chromatography conditions were modified using the ability of VersaPak cartridge to elute in opposite direction.

Flash Chromatography

The following step gradient conditions were used:

1. 20% acetonitrile – 200 mL

2. 60% acetonitrile – 200 mL

3. 100% acetonitrile – 100 mL (eluted in opposite direction)

100 mL fractions were collected manually and concentrated using rotor evaporator. Concentrated fractions were analyzed by HPLC.

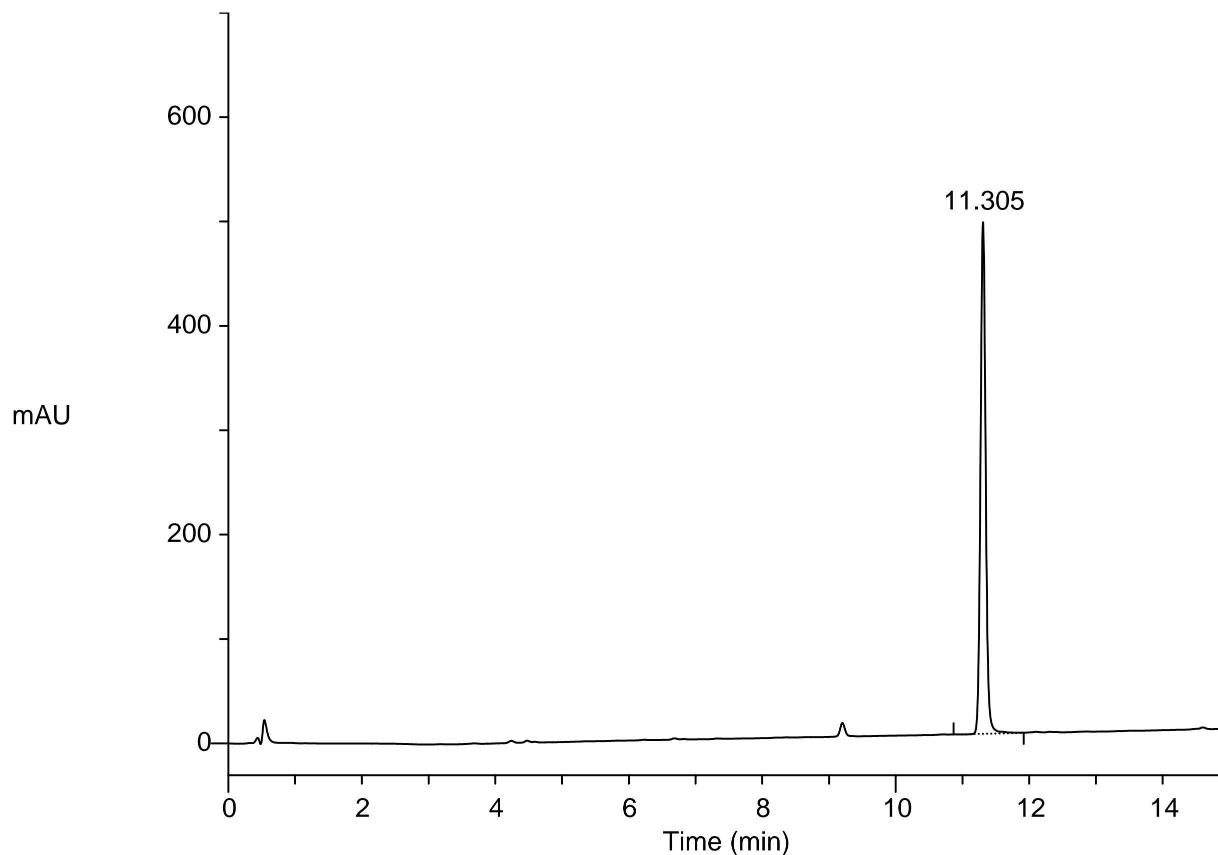
As a result, the compound of interest was concentrated in one 100 mL fraction of 100% organic solvent which makes future solvent evaporation much easier and faster. Purity and yield were the same as in a previous run.

Results

Gradient reversed-phase HPLC was used for flash chromatography method development. Based on a linear gradient HPLC, step-gradient conditions for flash purification was proposed. Due to acidic nature of purified peptide both HPLC separation and flash purification were carried out at neutral conditions (acetonitrile/water gradients). Three-step gradient allows to separate 10 mg of crude peptide and to obtain 85% yield and 95% purity of compound of interest within 20 minutes. Application of RevElution decreased elution volume for peptide of interest and allows to concentrate it faster.

Results

Figure 2. HPLC of Purified Peptide



Flash chromatography system equipped with C18 cartridges is a cost-efficient alternative to preparative HPLC for purification of synthetic peptides and other small molecular weight compounds.

References

1. C.Edwards, L.A.Lawton, S.M.Coyle, and P.Ross., J.Chrom., 734(1996) 163-173.
2. L.A.Lawton, J.McElhiney, C.Edwards, J.Chrom., 848 (1999) 515-522.
3. Expasy molecular biology database www.expasy.ch