HPLC purification of peptides and miniature proteins

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I. Introduction

High performance liquid chromatography, commonly known as HPLC, has a variety of applications in the chemical biology research laboratory. This protocol provides some basic background theory, some tips for getting ready to use the HPLC for your particular purification, and guidelines for doing an HPLC purification using our (fill in brand name) instrument. Click here to refer to the HPLC glossary for definitions of many of the terms used in the text.

Chromatography is a general analytical technique used to separate a mixture into its individual components. You should already be familiar with thin layer chromatography (tlc), which is used in organic chemistry to separate molecules based on structural differences. The individual components, or analytes, can then be analyzed free of interference from the other components. In chemical biology, individual analytes, such as peptides, are often chromatographically purified for use as a functional tool (e.g., binding to another molecule, enzymatic activity). High performance liquid chromatography (HPLC) is a method used to analyze and separate liquid samples. The separation apparatus is coupled to a UV detector to characterize the analytes as they are separated. In chemical biology laboratories, HPLC is considered indispensable for the purification of peptides (synthesized manually or automated with a synthesizer) and other small to medium-sized organic molecules.

Fundamentally, HPLC consists of passing a liquid sample (mixture of components, e.g. a crude peptide synthesis which will typically contain contaminants from the synthesis reagents, various truncated forms of the peptide, etc.) through a column under high pressure. This mobile phase passes through the material in the column, which is called the stationary phase. The analytes passing through the column interact at different rates between the mobile and stationary phases, primarily due to different polarities of the analytes. The analytes that interact least with the stationary phase or interact most with the mobile phase will exit the column faster. Propagated along the length of the column, these repeated interactions result in a separation of the analytes. (See Diagram 1.) Mixtures of various analytes can be analyzed by changing the polarities of the stationary phase and the mobile phase.

There are many types of HPLC columns developed for specific applications. The right choice of column is critical for obtaining good HPLC results. Column choice is governed by characteristics of components in the mixture we wish to separate. For example, we can separate components based on size, charge, hydrophobicity, aromatic character, even chirality. Variable factors include the polarity of the stationary phase, column dimensions, and pore sizes (which can be varied to allow certain sized analytes to pass through at different rates). Another variable that impacts the efficiency of the HPLC separation is the polarity of the mobile phase. Multisolvent delivery systems change the polarity of the mobile phase over the course of an HPLC run, at a rate that defines the "gradient" (e.g., 20% Buffer B to 100% Buffer B over 60 minutes). The use of a gradient improves the separation of analyte mixtures of varying polarities.

Typically, the stationary phase in an HPLC column is prepared by reacting an organochlorosilane with the reactive hydroxyl groups on silica. The organic functional

group is often a straight chain octyl (C-8) or octyldecyl (C-18) hydrocarbon. When the stationary phase is polar (silica or alumina) and the mobile phase relatively less polar (n-hexane, ethyl ether, chloroform), this is referred to as 'normal-phase chromatography.' An example of normal phase chromatography is a silica gel "flash" column, often used in organic chemistry to separate relatively non-polar water-insoluble organic compounds. Flash columns are used for the purification of synthetic b-amino acids. When the mobile phase is more polar than the stationary phase (as is the case with a C-8 or C-18 bonded phase), this type of chromatography is called 'reversed-phase chromatography.' Reversed-phase chromatography separations are carried out using a polar aqueous-based mobile phase mixture that contains an organic polar solvent such as methanol or acetonitrile. Because of its versatility, reversed-phased chromatography is the most frequently used HPLC method. Applications include non-ionic compounds, polar compounds (such as peptides), and in certain cases ionic compounds.

Analytes exiting the column can be detected by refractive index, electrochemical, or ultraviolet-absorbance changes in the mobile phase. The detector measures a signal peak as each analyte leaves the column. The signal intensity corresponds to the amount of analyte leaving the column, and can yield quantitative data when compared to a known amount of that particular analyte. The time it takes for the peak to show up, known as the retention time, is characteristic of a particular compound and thus enables identification of the peak of interest. Our HPLCs use a photodiode array detector (PDA) to continuously scan various wavelengths of the UV spectrum. As an analyte peak is detected, the UV spectrum is recorded. Over time, this compiled output yields a time-based plot called a chromatogram

The mechanics of the HPLC system are controlled by Windows-based software on a PC. This software controls the gradient of the mobile phase, the solvent flow rate, mobile phase pressure, and measures the signals produced by the detector. A specific HPLC protocol is stored as a method, the parameters of which can be adjusted as necessary. Finally, the results of your sample run can then be interpreted and printed in a variety of report formats.

(Adapted from http://www.gmu.edu/departments/SRIF/tutorial/hplc/hplc2.htm; http://www.laballiance.com/la_info/support/hplc3.htm; see also Waters website **http://www.waters.com/WatersDivision/ContentD.asp?ref=JDRS-5LTGBH.)**

II. HPLC Purification of Peptides (a and b)

A. Very Important Note

Everything that goes into the HPLC must be filtered first, through a 0.45 mm or 0.2 mm filter and special glassware to remove particles that can get caught up on the column and interfere with absorption and separation. This includes your buffers and your sample. Omission of this step can result in damage to the instrument.

B. Sample preparation

The crude peptide, prepared by manual or automated synthesis, will be supplied as a lyophilized (dried by freezing in a high vacuum) substance. For b-peptides, the sample is dissolved in 50% H_20/50% CH_3CN (or a range of others; solvent selection depends on solubility of the sample). Filter your sample.

C. Buffer Preparation

Buffer A and Buffer B are prepared according to the following recipes:

Buffer A	Buffer B
80 mL CH_3CN	3200 mL CH_3CN
3920 mL H_20	800 mL H_20
2.4 mL TFA*	2.0 mL TFA*

Safety precaution: Trifluoroacetic acid (TFA) is highly corrosive and causes severe burns when inhaled or upon contact with skin. This chemical should only be handled in the fume hood while wearing safety goggles, gloves, and protective clothing.

Filter your buffers, using the designated glassware and following the specific instructions provided by your TA. This can be done prior to use and buffers stored at room temperature until you are ready to use the HPLC.

D. HPLC Operation

Your TA will provide specific instructions pertaining to the use of the HPLC. Typically, a run starts by attaching your buffers and washing the column (100% Buffer B for 5-10 minutes). Next allow the column to re-equilibrate to conditions that will start your run. For a run with a gradient of 20% Buffer B to 100% Buffer B, this means allowing about 5-10 minutes for the starting conditions for injection to be achieved (that is, to get the entire column in 20% Buffer A).

Once a specific separation method is specified, you may review the parameters such as pump flow gradient, run time, and the PDA setup (acquisition). On some instruments, you will need to specify the lamp used for detection. Your TA will supply the details for the instrument you are using.

When making an injection, choose the amount based on the type of column you are using and the approximate amount of your sample. For a- and b-peptides, the following general guidelines apply:

Column scale	***Amount peptide per injection***
Analytical	Up to 0.01 mg
Semi-preparative	Up to 0.05 - 0.1 mg
Preparative	Up to 0.1 – 0.5 mg

Use either a glass syringe or a disposable plastic syringe fitted with a luer lock needle (only use flat-tipped needles). Before drawing up your sample, wash out the syringe several times with Buffer B. Draw your sample into the syringe, then carefully remove ALL bubbles from the sample by inverting the syringe, tapping gently, and expelling air until liquid just appears at the needle tip. Load your sample as instructed by your TA.

You will want to adjust the view on the PC screen for convenient monitoring of the run, which means selecting the appropriate wavelength(s). For a- and b-peptides, 214 nm (the absorption frequency of peptide bonds) and 280 nm (the absorption of tyrosine and tryptophan) are recommended. Notice the retention times listed (in minutes) at the bottom of the graphs as well as in the status bar at the top of the screen (this may vary depending on the software used; your TA will clarify this). You will need to record the retention times as you collect peaks so you can correlate your fractions with peaks on the chromatogram.

For the first injection of a peptide you've never purified before, you will need to carefully analyze the output. To do this, label a set of 15-20 tubes (15-ml conical vials usually work; you may want to do this ahead of time and loosen the caps so they are

ready for collecting peaks as they come off the column. Once you have collected all the relevant peaks from the first injection, you will analyze them by mass spectrometry and determine which fraction or fractions contain your peptide by looking for its molecular weight (calculated in advance). Matching these fractions to their corresponding peaks will give you the retention time for your molecule. At this point, further injections will be simplified as you can accurately predict the retention time of your sample, and you'll know where to expect the peak containing your molecule.

The first peaks that come off the column (after 3-4 minutes dead time for the semi-prep column, 5 minutes dead time for a prep column) represent a variety of leftovers from the synthesis (usually incomplete removal of reagents during wash steps). Once you get beyond this point you should collect every peak as it comes off the column, noting the retention time (for example: 11.23-11.5) for each numbered tube. Try to separate shoulders from main peaks, and isolate peaks that appear within multiple peaks. Keep in mind that the method you choose will impact the appearance of the chromatogram, and hence your ability to collect a pure, isolated fraction. For example, a longer method will give better resolution, but broader peaks. You will get better at this technique with practice, and bear in mind that it usually takes at least two passes through a column to purify a crude peptide synthesis.

III. HPLC Glossary

For a comprehensive list of terms, see http://www.waters.com/watersdivision/images/aboutus/hplcglossary.htm.

Analyte – One of the components in a mixture to be chromatographically separated (e.g., the synthetic peptide, a reagent leftover from synthesis)

Chromatography – A separation technique based on the differential distribution of the constituents of a mixture between two phases, one of which moves relative to the other.

Chromatogram – The electronic result of a chromatographic separation that plots the UV detector signal output versus retention time. It is represented as a series of peaks.

Column – A tube containing the stationary phase. The stationary phase differentially interacts with the sample's constituent compounds as they are carried along in the mobile phase.

Fraction – A sample collected from the instrument after it has flowed through the column and passed by the signal detector.

Gradient – The change in mobile phase composition over time. This can be continuous or stepwise.

Mobile phase – The solvent that moves the mixture of compounds through the column.

Resolution – The efficiency of separation of two or more peaks. A well-resolved peak should be symmetric, touch the baseline, and not interfere with any other peaks.

Retention time – The elapsed time between injection of a sample and appearance of a peak maximum.

Stationary phase – The immobile phase in the chromatographic process. In HPLC, this is a solid material packed inside a column.

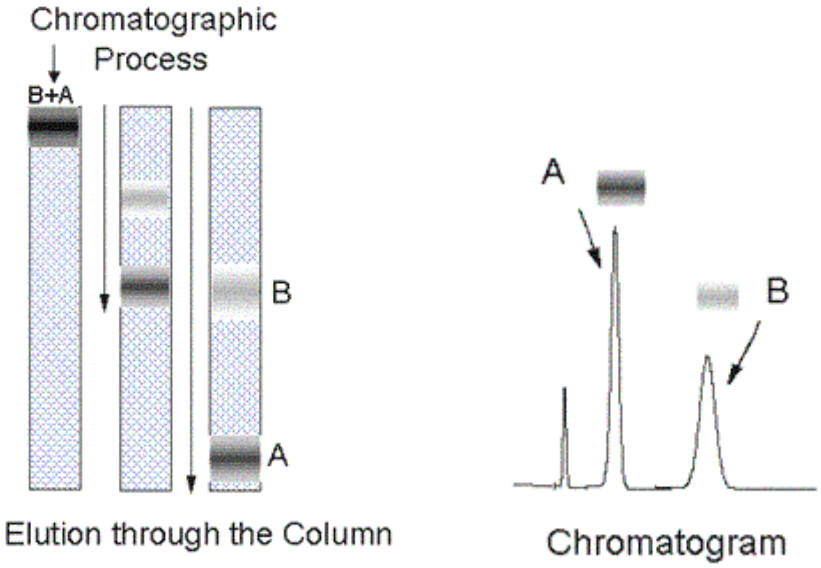

Diagram 1
B + A represents a mixture of analytes to be separated. **A** moves faster through the column than **B**, and will therefore have a shorter retention time. The small peak to the left of **A** represents unwanted material such as degradation products or leftovers from synthesis. This material often shows up as several peaks, sometimes larger than the product peak(s), at an early point in the separation.