Biology | Biochemistry

High Performance Liquid Chromatography (HPLC) of Proteins

General Aim

To separate and detect amino acids in a tissue.

Method

HPLC - reversed phase column chromatography.

Learning Objectives (ILOs)

- To prepare the mobile phase by vacuum filtration.
- To prepare protein samples by acid hydrolysis prior to injection.
- Perform online OPA/FMOC sample derivatization/ injection.
- To list the parameters set for an HPLC run.
- To visualize results of HPLC on a chromatogram.

Theoretical Background/Context

HPLC is a chromatographic method used to separate the components of a mixture. The typical design of an HPLC experiment usually involves these elements.

a. A filtered solvent reservoir for mobile phase.
b. Degassing system to prevent bubbles in mobile phase.
c. A pump to maintain constant flow of mobile phase despite the back pressure applied by the resistance of flow through the packed column.
d. An injector to inject samples. This can be manual or automatic.
e. A column (solid phase). Length, internal diameter and stationary phases are to be chosen.
f. A detector/data system to plot the chromatogram. includes UV, diode and fluorescent detectors.

Samples are forced to flow under high pressure through the column (solid phase). Solvents (mobile phase) allow the flow of the sample into the column, where it is separated into its components according to their interaction with the solid phase. components are detected and recorded on a chromatogram. The resolution of various components is determined by the extent of interaction between the solute components and the stationary phase.

Theoretical Background/Context (Cont')

In normal phase chromatography, the mobile phase is non polar while the stationary phase is polar. In reversed phase chromatography, the mobile phase is polar while the stationary phase is non polar.

Principle of Work

Method development for an HPLC experiment usually follows these steps:

1. Choosing the mode. In this experiment we choose the reversed phase chromatography.
2. Choosing the column and column packing dimensions. For example in this experiment, it is suitable to use a reversed-phase column Agilent ZORBAX Eclipse Plus C18 columns and recommended guard cartridges.
3. Choosing the stationary phase chemistry.
4. Choosing the mobile phase solvents and their pH.
5. Running initial isocratic or gradient experiments to define boundary conditions and optimize the experimental conditions.

The general steps for Amino Acid analysis include:

1. Preparation of HPLC mobile phases: filtration with vacuum pump.
2. Preparation of amino acid standards.
3. Preparation of Internal Standard (IS) stock solution.
4. Tissue sample preparation: sample is minced and prepared using perchloric acid for protein separation, then lyophilized. The dried protein then undergoes acid hydrolysis using 6 M HCL.
5. Online ortho-phthalaldehyde /9-fluorenylmethyl chloroformate (OPA/FMOC) precolumn derivatization is performed.
6. Setting the parameters of detection.