Method Development for Vitamin B12 Analysis

Introduction:

High-performance liquid chromatography (HPLC) is a chromatographic technique used for efficient separations and analyses of a mixture of components in a liquid phase. A sample in solution is introduced into a column packed with chromatographic media (stationary phase). Differential partitioning of the mixture components between a stationary phase and a mobile phase (the elution solvent) takes place, resulting in differential retention and elution of a mixture of components from the column. The polarity of the stationary phase is different depending on the length of the non-polar components on the silicone backbone. A C18 RP column is less polar for example than a C12 RP column. The use of an ultra high-pressure pump to move a sample through a tightly packed column is the distinguishing feature of HPLC and UHPLC.

There are several modes of liquid chromatography. These are generally defined according to the specific types of interactions responsible for separation. In this experiment, we will employ reversed-phase (RP) liquid chromatography, where polar mobile and non-polar stationary phases are used. Here, the separation is based primarily on hydrophobic interactions between a sample and a stationary phase. Mobile phase composition (pH, organic solvent used, % organic solvent) is the most important factor in optimizing reversed-phase LC separations, as it allows varying the degree of retention of a given analyte on a particular column.

Pre-Lab Questions:

1) What are the physical difference between C18 and C12 RP HPLC columns?
2) How could you increase efficiency of RP HPLC separations?
3) When would you use HPLC in your research/analysis?
4) What is difference between solid core column and non-solid core HPLC columns?

Materials:

HPLC grade methanol, C18, C8, C12 and other modified C18 columns. Varian Prostar HPLC system with diode array detector.

Experimental:

The purpose of this experiment is to develop a HPLC methodology for measuring vitamin B12 in a medication and/or natural product. The solvents that will be used include water, and methanol. Any solvents that you make should be mixed, if necessary, and then filtered before use through a 0.45 um filter. You can use either an isocratic system or a gradient. A Vitamin B12 standard is available to use to work out the methodology. A number of RP HPLC columns are available for you to try. You should try at least two different columns for your separation method. Vitamin B12 absorbs light at wavelengths 254 and 361. The HPLC is the Varian Prostar with a diode array detector and a binary pump system. You will have to look in the literature for some ideas on what the best methods are. The galaxie software should be open. Set the light sources on under the 335 icon, and the flow rate and initial solvent concentration under the 210 icon. The columns need about 6 to 10 solvent column volumes to equilibrate to the

starting solvent concentration. Make up your method under '"new method"'. All changes are under "control" icon. Set the solvent program, and absorption wavelengths. The solvent program should star at the initial solvent concentration than ramp up to the highest concentration over an allotted time period. The solvent then should be ramped down to the initial solvent concentration for re-equilibration of the column, and stay at this concentration for at least 6 column flush volumes before injecting next sample. All injections should be done using the sample sequence system. Under "file" open "new sequence" and fill in table. You need to add method, sample name, suffix, vial number, number of injections and injection volume of 10ul.

Method Development:

The purpose of this experiment is to develop a method to measure Vitamin B12. You will need to dissolve a vitamin sample (pill) in water and then filter out the filler material. Use a syringe filter with a .45um filter. You will need to determine the solvents and column that will give you the best separation. You will probably use a fairly large gradient (maybe 5% methanol to 80% over 15 min.) to see if you can get separation and then find where best ratio of solvents and time are. You will try a C12 column first and then one or more C18 column(s). When you find a column that separates your compounds you will optimize the solvent gradient (or determine if isocratic method could work). You can start your gradient at a higher methanol content if there is excessive time before sample comes off the column. You may be able to decrease the run time if a lot of time is left after sample elutes off column. When you have best method you can run a standard sample to determine the approximate concentration of your pill for B12.

You should be conscious of keeping the method as short as possible to conserve solvent and to allow analysis of multiple samples. Since you have limited time to carry out this procedure you must come to the laboratory well prepared and have your procedures written in your laboratory notebook. Whether your experiments are successful or not this is the information you will have to write up for your experiment so the more combinations you try the better and more comprehensive will be your write-up. Make sure you record all changes you have made, columns you have used, etc. so you can include these in your report.

Data:

Your data will include all the methods results that you tried plus analysis of a commercial Vitamin B12 pill. If a method does not work it should still be part of the trial since you are demonstrating to the reader which methods work and which don't and possibly to the best of your ability why they work or not.

Discussion:

Your discussion will include your method and compare to other methods (literature search) that you have found and also compare any methods you tried and whether they worked or not. The reasons the you changed methods should be included. This experiment should help you prepare for future experiments if you go into graduate studies.

Conclusion:

The conclusion should be a summary of your results and your methodology recommendations, whether your separation worked or not.

Final Questions:

1) Why is HPLC the best instrument for your method?
2) What other instrument would work for this analysis?
3) Could you use Uv-Vis spectroscopy to measure B12? Explain.
4) What are a few problems associated with measuring this vitamin in medication or food stuff?