Name _____

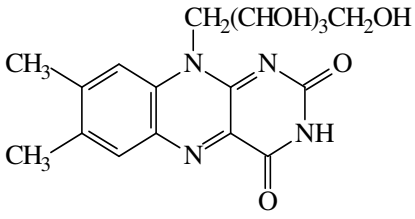
Experiment 2C

Integrated Laboratory Experiment

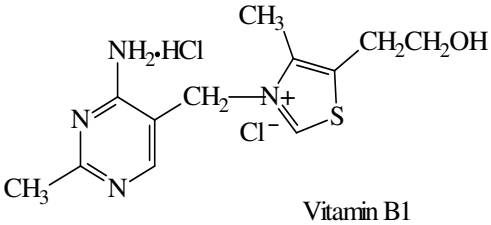
DETERMINATION OF RIBOFLAVIN: A COMPARISON OF TECHNIQUES

PART C. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The purpose of this experiment is to introduce you to high performance liquid chromatography (HPLC). You will use HPLC to analyze the concentrations of vitamins B1 and B2 (see Figure 1) found in the commercial vitamin tablet. This part of the project covers optimizing the gradient profile for the HPLC mobile phase composition to get good separation between the peaks for vitamin B1 and B2, running several standards of these vitamins, and constructing a calibration curve for each vitamin, and then running samples of the vitamin pill to find concentration for each one. Once the concentrations for the sample solutions are determined from the standard curve, the original concentration of each vitamin in the tablet (mg/tablet) are found by back calculation as you have done in expt 2A and 2B. The vitamins are detected at their optimal wavelengths in the UV.



Riboflavin
Vitamin B2



Vitamin B1
Thiamine chloride, hydrochloride salt
pK 3.4 (thiazole) and 4.8 (free amino)

Figure 1. Structures for Vitamin B2 and Vitamin B1.

A goal for HPLC methods is to achieve satisfactory separation of the analytes in a convenient period of time. The first step in method development is to optimize the mobile phase composition by adjusting proportions for components in the mobile phase by pumping different amounts of each over time, call running a gradient. The analytes and the stationary phase in reversed phase HPLC are hydrophobic, and the mobile phase is hydrophilic (water to which an adjustable amount of an organic co-solvent has been added.) If the amount of organic co-solvent is too high, the analytes will be highly soluble in the mobile phase, insufficiently retained by the stationary phase, pass rapidly through the column and not be satisfactorily separated. If the amount of organic co-solvent is too low, the analytes will be virtually insoluble in the mobile phase, strongly retained on the stationary phase, and the

retention times will be inconveniently long. The challenge is to find the optimum between these extremes. In this experiment the organic co-solvent is acetonitrile and the stationary phase is a Poroshell-C18.

A. Experimental (Report details are in part B.)

An instructor will guide you through the use of Agilent 1260 Infinity HPLCs in GbAd318. These HPLC units.are ~$40K each and typical instruments found in many research labs and industrial QC/QA labs. These units include a high pressure quaternary pump, vacuum degasser, an injection valve with either a 5 or 20 μL sample loop, a reverse-phase column - C18 column (2.7 μm particles, 50 mm long, 4.6 mm i.d.). Two systems by the door have variable wavelength UV-Vis detectors and the third system by the windows has a diode array detector (DAD). All three systems are operated using software called ChemStation. The inlet of the pump is attached to a valve which is programed using ChemStation to make a gradient of usually two to four mobile phase mixtures stored in the bottles on top of the solvent cabinet. The system can also be operated in an isocratic mode in which the composition of the mobile phase does not change over the course of a run. A special blunt tipped needle on a 50 or 100 μL syringe is used to flush and then load the injector loop in one step.

Standard Solutions. Two standard solutions each containing both vitamins are available in the lab:

1. 10 μg/mL vitamin B1 and 10 μg/mL vitamin B2 in millipore water

2. 20 μg/mL vitamin B1 and 20 μg/mL vitamin B2 in millipore water

Sample Solution

For the commercial vitamin samples, use the three "Y" sample solutions prepared in part 2A. **Filter about 1 mL of each Y solution into a sample vial using a 0.22-μm nylon disc filter fitted on a 3 mL plastic syringe and label the vial.** *Store filtered test solutions in the dark until ready to run.*

Overview of experiment including mobile phase gradient optimization and running samples.

1. First carry out a separation of the 20 µg/mL mixed vitamins B1 plus B2 standard provided for you in the lab using two different gradients for the mobile phase, Gradient 1 and Gradient 2. Each gradient program is setup so that there are different proportions of pH 7.0 buffer (component A) and acetonitrile (component B) automatically mixed for the mobile phase over the time for a run. This changes the polarity of the mobile phase over the course of a run. Your first job is to find the gradient that gives the better separation of the peaks and possibly a shorter analysis time for the **20 µg/mL** mixed standard.

2. Your second job is to run the 25 **µ**g/mL riboflavin standard that contains only riboflavin to confirm the identity of the chromatographic peaks for riboflavin and thiamin and determine their retention times.

3. Your third job is to determine if the calibration is working by running the second mixed standard (**10 µg/mL each vitamin).** Check that the ratio of peak areas for the two vitamins of interest are proportional to their concentrations in the two standard solutions (i.e., 2:1).

4. Once the calibration is working, your fourth job is to run the three filtered Y pill solutions with the better mobile phase gradient.

5. Time permitting: each group should run one filtered Y pill solution on system #3 by the windows that is equipped with a DAD detector even if you are not using this system for the complete analysis. Obtain one copy of the 3D chromatogram display at the three different wavelengths used on system #3. If your group is using one of the other systems (#1 or #2), for your complete analysis, you do not need to run more than one Y sample on system #3. If the instrument is not available, or time is limited, see the 3D chromatogram posted on the course web page and use this to answer the question in your report.

Instrument Setup and Conditions and Running Experiment:

1. Log on as Science/GBAD318/ with psswd Chemistry and the server is Science. Launch "HPLC-PCXX (online)" where XX is the number of the PC at your instrument using the same science/CHEM461/chemistry/.

2. Select "Method and Run control" on the lower left table.

3. Select the "ON" buttons to turn on both the quaternary pump and the UV lamp.

4. In the top menu go to Method → Load method → CH461 vit_gradient 1 (it is located in C:/Chem32/1/Methods).

5. Check instrument settings according to Table 1 below and - use "Gradient 1" for the starting mobile phase composition. Check in the method to make sure the settings match the %A and % B in Table 1 below for Gradient 1. You can see the percentages by clicking in the pump window and select "method", but don't change any of the settings without checking with the instructor first.

6. From the top menu go Run Control → Sample Info. Specify your file name and sample name. Make sure that the subdirectory is specified as "CH461". **Repeat this step before each run**. **Otherwise your run data will be overwritten.**

7. Load the 50 or 100-µL HPLC syringe **(check that this syringe needle has a blunt tip)** with the 20 ug/mL mixed vitamin standard solution, making sure that there are no air bubbles in the syringe and that the syringe is completely filled (50 or 100 uL). .

8. With the injector valve in the **load** position, flush and fill the sample loop all at one time by carefully pressing the plunger all the way (watch for air bubbles that collect on the tip of the plunger - don't add the air bubbles to the sample loop) . Let go of the syringe and leave the syringe in the valve and **carefully** rotate the injector valve to the inject position **(it only goes about 1/3 of the way clockwise and stops-don't force it).** This action will trigger ChemStation to start the run. After about 30 s into the run, turn the valve back to load and

remove the syringe. It can be rinsed and setup with the next solution. Rinse well as this is a very sensitive technique. The run will automatically stop at 5 minutes and go through a 2.0 min post-run to flush the column and return to the starting mobile phase composition and the program will automatically re-zero the absorbance on the starting mobile phase stream. A report will be generated automatically after the run. If needed you can print the report by going to the printer icon on the menu in the middle of the screen on system 1 and 2 ("identify peaks, calculate runs, print); on system 3 go to Data Analysis, on the top menu, and select Print report (short). Get a hard copy from the printer in the lab. The chromatogram can also be copied to the clip board and pasted into word for example.

9. To change to Gradient 2, go to Methods on the top menu, Load CH461_Vit_Gradient_2. Check the timetable in the method to make sure it matches with Gradient 2 Table 1. below.

10. Run the Y samples using the better gradient . Check that the areas for the vitamins fall within the standards. Some of the Y solutions you analyzed in part 2A can show low concentrations of B2, in these cases your samples may fall below the 10 mixed standard but should be greater than zero. Time permitting, run one of your Y solutions on system 3 to get the 3D chromatogram (wavelength vs. time vs. absorbance).

11. After completing all the runs go to C:/Chem32/1/Data/CH461 and create a folder with your group number or initials and move all folders with created reports and all data for your group there.

Table 1. Gradient Settings and Wavelength Settings

Mobile Phase A	pH 7.0 K_2HPO_4 (aq)		
Mobile Phase B	HPLC grade acetonitrile		
	Time, min	% A pH 7.0 buffer	% B acetonitrile
Gradient 1	0	100	0
	3	65	35
	4	65	35
Gradient 2	0	80	20
	3	65	35
	4	65	35
Flow Rate	1.00 mL/min		
Stop Time	5.00 min		
Postrun time	2.00 min		
detector wavelength (systems 1 & 2)	262 nm		
detector wavelengths on system 3	215, 262 and 450 nm		

3D Chromatogram only at HPLC 3 by the windows:

Time permitting, each group should run one Y sample solution on the HPLC with diode-array detector (DAD) by the windows. In the Method for UV-lamp select 3 wavelengths - 214, 262, 450 nm (bandwidth 4 nm) with reference 550 nm (bandwidth 10 nm). Identify B1, B2 vitamins and if possible other B vitamins present in the tablet solution. Use information on vitamin absorbance (see Table 2 below) to identify peaks. Note that the vitamins are expected to elute in the order they are listed in the table. To obtain a full spectrum in 190-500 nm range select a chromatogram of interest, click To obtain a full spectrum in 190-500 nm range select a chromatogram of interest, click
 then 

"Select spectrum at any time position" 

Table 2. Absorbance Maxima for B Vitamins

SI No:	Vitamin	Absorbance max, nm
1	calcium pantothenate	205
2	pyridoxine	220
3	niacinamide	214
4	thiamine	232
5	folic acid	280
6	biotin	205
7	cyanocobalamin	214
8	riboflavin	268, 445

Calibration and Sample Analysis (**optional** after lab not during lab)

1. Load ChemStation offline instead of online.
2. Highlight "Data Analysis" in lower left table of the main screen. Open the file of interest by navigating in the upper left of the screen.
3. Click on 

to work in the calibration mode.
4. To create a calibration table and build calibration curves for known compounds click on "New calibration table from current chromatogram". Label known compounds in the table and specify their concentration.

5. Select another standard run and add it to the table by clicking on  "Add new level on current chromatogram". Click "OK" to save the calibration table.
6. To print the resulting table and calibration curves go to File → Print → Calib. Table + Curves

B. Report

The report should include the following (no introduction is required):

1. Scientific Abstract. Summary of results with error estimates.

2. All chromatograms with proper labeling of sample and conditions (flow rate, λ, gradient, sample loop volume, etc.).

3. A table giving the run number, the retention times and the raw area numbers for the peaks for vitamins B1 and B2 for all chromatograms. Use three significant figures for the peak areas in scientific notation.

4. Give a brief explanation of why you picked the mobile phase gradient that you did for the final analysis of the sample. Include your rationale in terms of the polarity of mobile phase over the course of the run, polarity of the column, polarity of the components, and anything else you think is important in this study.

5. In Excel produce plots for the two calibration curves, one for riboflavin and one for thiamine based on peak areas and on peak heights. Use point (0,0) as a third standard data point and report the slope and intercept results of linear regression using Excel for each vitamin curve. If available you can use the automatic calibration functions in ChemStation.

6. Use the calibration curve equation to determine the individual values in μg/mL of both vitamins for each run of the Y test solutions. Present this information in a table. Based on the whole tablet weight, and the actual weight of tablet dissolved to make solution X, plus the dilution of solution X to make the test solution Y, perform a "back calculation" and find the mg/pill value in the original vitamin pill for each of the three samples. Finally report a mean and RSD for each of the two vitamins that you analyzed for in the pill. Include sample

calculations.

7. How do your results for mg of thiamine per tablet compare to that expected? What did you learn from the 3D chromatogram run on the third system equipped with the DAD detector? Explain briefly.

8. Use a proper table and present a comparison of the tablet analysis for riboflavin to that reported by the manufacturer and to those results obtained in experiments 2A and 2B. Remember to include the individual values and mean value and standard deviation of the milligrams of riboflavin per tablet with each technique.

9. Suppose you work in a research lab helping a team to study the effects of the riboflavin on heart muscle. Your first assignment is to analyze five different brands of commercial vitamins for riboflavin and your supervisor needs this information in one week - renewal of a multi-million dollar grant is riding on the accuracy of your analysis! To improve statistics, you decide to analyze four bottles of each brand and four tablets from each bottle, in triplicate. How many individual runs are required to do this number of samples? Which of the three techniques that you studied ----(molecular absorption 2A), molecular fluorescence (2B) or HPLC (2C) ----would you use for this project and briefly state why you think this.