**PURDUE UNIVERSITY INSTRUMENT VAN PROJECT**

## HOW MUCH ASPIRIN, ACETAMINOPHEN, AND CAFFEINE ARE IN YOUR PAIN RELIEVER?

### USING HPLC TO QUANTITATE SUBSTANCES
(Revised: 1-13-93)

INTRODUCTION

Headache, sore muscles, arthritis pain... How do you spell relief? Pain serves the useful function of alerting you when some component of a physiological system has gone awry. Ideally, the pain can be alleviated by removing the underlying cause. In many cases, however, the stimulus of pain is either not easily defined or is not readily removed; therefore, it is necessary to treat pain as a symptom.

As a result, Americans spend millions of dollars annually on NON-NARCOTIC ANALGESICS sold as over-the-counter pain relievers. Pharmaceutical companies go to great lengths to convince you that Anacin® works twice as fast or that you can "Nupe it with Nuprin®". But the bottom line is that there are only three non-narcotic pain relievers which are considered safe enough to be sold over-the-counter.

The oldest of these is aspirin, which has the chemical name acetylsalicylic acid. Salicylates were discovered following the extraction of a naturally occurring substance, glycoside salicin, from willow bark. The original compounds were found to have medicinal value but also some side effects. Further investigations led to aspirin, an ester derivative of salicylic acid, which was first marketed commercially by the Bayer Company in Germany in 1899.

Acetaminophen became available around 1950. While acetaminophen relieves pain and reduces fever, it does not relieve stiffness, redness and swelling of arthritis as aspirin does.

Ibuprofen was first used predominantly for its anti-inflammatory activity. It was first released in 1984 for over-the-counter use and has now replaced some of the market share of aspirin-based compounds.

Caffeine is not a pain reliever, although it is sometimes found in these products. Caffeine acts by stimulating the central nervous system, and when present in some of these products may provide additional relief of headache pain or faster relief.

This is both a qualitative and quantitative experiment and in it we will determine which and how much of the three compounds (aspirin, acetaminophen, and/or caffeine) are present in a given relief product. (Ibuprofen can also be used, but we will not quantitatively analyze it since we do not have a pure standard of ibuprofen.) In order to identify the compounds, we will need to separate them. To do this we will utilize a High Performance (Pressure) Liquid Chromatograph

or HPLC.

Chromatography consists of two phases: the mobile phase and the stationary phase. In our experiment our mobile phase is the solution containing 30% methanol, 2% acetic acid, and 68% distilled water. The stationary phase contained in the column consists of silica beads coated with "C-18" chains. In other words, the surface of our stationary phase consists of hydrocarbon chains containing 18 carbons. For example:

(silica bead) -$CH_2$-$CH_2$-$CH_2$-$CH_2$-$CH_2$-$(CH_2)_{10}$-$CH_2$-$CH_2$-$CH_3$     ←surface

One important property of our phases is their relative polarity. Our mobile phase is much more **polar** than our stationary phase. Why do we care about the polarities? Let's imagine that we have two different type of molecules at the start of the column, molecule A and molecule B. Now if A is more polar than B, A will be attracted to the mobile phase than B. Since A is more attracted to the mobile phase, it will be moving with the mobile phase a larger percentage of the time than B will since B is more attracted to the stationary phase. We can see then that A will move through the column faster than B does. In other words, A would have a lower **retention time**. **Retention time** is the mount of time that a compound takes to pass through a column. By marking our chromatogram, we can determine the retention time by determining the time from the **solvent front** to the middle of our retained peak. The **solvent front** is the peak that occurs when the non-retained part of the injection first passes through the detector.

After passing through the column, the molecules in solution pass through a detector. Our detector measures how much the solution absorbs ultraviolet radiation with a wavelength of 254 nm. Solutions that contain greater concentrations of molecules that absorb at this wavelength will show larger "peaks". Unless we make a standard curve, though, we cannot look at our chromatogram and compare concentrations of different peaks by comparing the sizes of different peaks. The reason we cannot is that different compounds have different molar absorptivities (or a relative ability to absorb radiation) at 254 nm. In other words, a one molar solution of aspirin will have a different sized peak on a chromatogram than a one molar solution of acetaminophen.

One other problem we can run into is that some compounds may have similar retention times. For instance, in our lab, ibuprofen and aspirin have essentially the same retention time. Unless we run further tests on our unknown samples, we will only be able to verify that our unknowns contain either aspirin or ibuprofen. For this reason, your teacher may opt to only test for acetaminophen, aspirin, and caffeine.

In this lab, we will measure the quantity of aspirin, acetaminophen and caffeine in our unknowns. (We are not calibrating a curve for ibuprofen since we do not have a pure sample of ibuprofen.) In order to do this, we will establish standard curves for each of these compounds. A standard curve is determined by measuring the size of the peak corresponding to a certain concentration of a pure known sample. By determining the peak area of a range of

concentrations for a given compound, we can calibrate the concentration that would correspond to a given peak size for our unknown sample.

## PURPOSE

The purpose of this laboratory is to introduce the HPLC as a tool for identifying and separating compounds by measuring different retention times. We will also explore the HPLC as a quantitative instrument.

## MATERIALS

HPLC equipment
Mobile phase: [Provided by Chemobile]
        30% methanol   (300 mL methanol, 20 mL acetic acid, 680 mL deionized water - used for experiment)
        75% methanol   (750 mL methanol, 250 mL deionized water - used for storage)
Known standards
Pain relievers to serve as unknowns
Standard solutions
Several 100 mL volumetric flasks

## PROCEDURE

**\*Preparation of Stock Solutions\***

1.  Solid samples of the standards will be provided.  It will be necessary to make solutions of these solids.  Very small quantities of these solids will be needed.  In order to use a quantity of solid which can be recorded accurately on the balances we will make up a solution and then dilute it further with distilled water.  Begin by making up the following solutions in 1 L quantities:

    Caffeine:  0. 110 mg/mL
    Acetaminophen:  0.126 mg/mL
    Aspirin:  0.115 mg/mL

2.  These solutions then need to be diluted to make the stock solution.  Follow the directions

below:

**Caffeine Stock Solution**:  Using a pipet, transfer 30 mL of the caffeine solution into a 100 mL volumetric flask.  Fill the flask to the mark with distilled water.

**Acetaminophen Stock Solution**:  Using a pipet, transfer 10 mL of the acetaminophen solution into a 100 mL volumetric flask.  Fill the flask to the mark with distilled water.

**Aspirin Stock Solution**:  The aspirin solution does not need to be diluted.  Transfer about 50 mL of the aspirin solution into a beaker.

3.  To make a calibration curve for each of the standards a series of dilutions will be done. This will allow you to make a graph of known concentrations vs. peak areas.   The following series of dilutions needs to be done for each of the stock solutions: [Be sure to mark each of the volumetric flasks with the identity of the dilution!]

Dilution #1:  Transfer 10 mL of Stock Solution into a 100 mL volumetric flask.  Fill to the mark with distilled water.

Dilution #2:  Transfer 7 mL of Stock Solution into a 100 mL volumetric flask.  Fill to the mark with distilled water.

Dilution #3:  Transfer 4 mL of Stock Solution into a 100 mL volumetric flask.  Fill to the mark with distilled water.

Dilution #4:  Transfer 1 mL of Stock Solution into a 100 mL volumetric flask.  Fill to the mark with distilled water.

4.  Refer to **HPLC Instructions For Operation** for the injection procedure. NOTE:  ALL samples must be filtered prior to injection!

Inject your samples one at a time at the following AUFS* values:

* AUFS = absorbance units full scale.  Thus AUFS = 0.02 means that an absorbance reading of 0.02 would register full scale on the recorder.

Caffeine:  0.01 AUFS (Set the **AUFS Range** knob to this value)
Acetaminophen:  0.02 AUFS
Aspirin:  0.005 AUFS

5.  Run all samples allowing time for each sample to elute before making another injection. After you are finished remove the chart paper from the recorder.

6.  Label all peaks and then determine the area of each one.  Your teacher will instruct you to use either the "triangulation" method or the "cut and weigh" method.

A.)     **"triangulation" method**

i.)  Assume that the peak is a triangle.
ii.) Determine the relative peak area as :
        Area  =  height  x  width at 1/2 height
iii.)Multiply the relative peak area x the **Range (AUFS)** value to calculate the adjusted peak area

B.)     **"cut and weigh" method**

i.) Cut out the standard peak and record the mass in mg.
ii.) Cut out a 1 square cm piece of the chart paper and record its mass in mg.
iii.)Divide the mass of the peak by the mass of the $cm^2$ to get the relative peak area.
iv.) Multiply the relative peak area x the **Range (AUFS)** value to calculate the adjusted peak area.

7.  Calculate the concentration for each of the solution in the dilution process.

Sample Calculation for Caffeine Concentration:

The first caffeine solution contained 0.110 mg.mL caffeine.

Our **Caffeine Stock Solution** was a dilution of the above containing:

Finally,

caffeine dilution #1 is a 10:100 dilution of the stock solution:

8.  Use the Data/Calculation Table to record your measurements and calculations.

9.  On graph paper, plot the values from the Data/Calculation Table with the concentration on the x-axis vs. the adjusted peak area on the y-axis. Label both axes including units. Draw the "best fit line" through the points on the graph, using the origin as a point.

**\*Sample Preparation\***

1.  To test a commercial sample you must first make a solution of the cold medicine. Use the information given on the label to estimate a dilution for the sample.

    To test for each component try to dilute the sample so that you are in the following standard curve range:

| Component | Dilution Range (mg/mL) |
|---|---|
| Caffeine | 0.0033-0.00033 |
| Acetaminophen | 0.013-0.0013 |
| Aspirin | 0.012-0.0012 |

Here is an example of how to estimate the appropriate dilution:

According to label information, a 3.5 gram Alka-Seltzer Plus® tablet is supposed to contain 325 mg of aspirin. First, let's dilute 100 mg of the Alka-Seltzer Plus® tablet in 100 mL of distilled water.

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A dilution of 100 mg of the tablet in 100 mL of $H_2O$ would yield:

This is a little too concentrated for our standard curve range for aspirin. We will need to dilute this solution some more.

Let's transfer 10 mL of this dilution into a 1000 mL volumetric flask and dilute to the mark:

Diluting this solution 10:1000 would yield an approximate dilution of 0.0093 mg aspirin/ mL of $H_2O$ which would be in our standard curve range.

We are going to all this trouble in order to use our standard curve to calculate the concentration of the compounds in our unknown.

2. Now inject the samples you have prepared at **Range (AUFS)** value = 0.05.

   If the peak is too small, inject the sample again with the **Range (AUFS)** at the next lower value.

   If the peak is off scale, inject the sample again with the **Range (AUFS)** at the next higher value.

3. After you get an acceptable chromatogram for your samples, calculate the peak areas as you did earlier (Step 6).

**\*Identification of Compounds\***

Comparing **retention times** should give a good estimate as to the compound(s) in the pain reliever. To "confirm" the identification, we will "spike" the unknown sample with known standards.

For instance, if you want to confirm that you have a caffeine peak, combine a small amount of caffeine solution with a small amount of your sample solution. Inject the filtered sample and compare with the first injection of your sample. If you are correct, the peak that you identified should be noticeably larger.

Remember, in the case of the aspirin and the ibuprofen which have similar retention times, we can only confirm that we have aspirin and/or ibuprofen. Final confirmation can be done by

checking the label of the product.

**\* Calculation of Concentration\***

Locate your value from the calculated peak area of you cold compound on the y-axis of your standard curve graph.  Draw a horizontal line from your peak area value until it intersects the best fit line on the graph.  From the intersection of the horizontal line and the best fit line, drop a vertical line until it intersects the x-axis.  The intersection on the x-axis is the concentration of the sample that was injected, NOT the actual pill/ tablet.

In order to calculate the amount of the compound you will need to back calculate.
For example: (Continuing the Alka-Seltzer Plus® example)

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**Name** _____          **Identity of Pain Reliever**_____

**Class Period**_____

### DATA/ CALCULATION TABLE FOR STANDARDS
### (Triangulation Method)

| Peak Identification | Peak Height (cm) | Width at 1/2 Peak Height (cm) | Relative Peak Area (cm$^2$) | Range (AUFS) | Adjusted Peak Area (cm$^2$) |
|---|---|---|---|---|---|
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### DATA/ CALCULATION TABLE FOR PAIN RELIEVERS
### (Triangulation Method)

| Peak Identification | Peak Height (cm) | Width at 1/2 Peak Height (cm) | Relative Peak Area (cm$^2$) | Range (AUFS) | Adjusted Peak Area (cm$^2$) |
|---|---|---|---|---|---|
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**Name** _____          **Identity of Pain Reliever** _____

**Class Period** _____

## DATA/ CALCULATION TABLE FOR STANDARDS
### (Cut and Weigh Method)

| Peak Identification | Peak Mass (mg) | Mass of $cm^2$ Piece (mg) | Relative Peak Area $(cm^2)$ | Range (AUFS) | Adjusted Peak Area $(cm^2)$ |
|---|---|---|---|---|---|
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## DATA/ CALCULATION TABLE FOR PAIN RELIEVERS
### (Cut and Weigh Method)

| Peak Identification | Peak Mass (mg) | Mass of $cm^2$ Piece (mg) | Relative Peak Area $(cm^2)$ | Range (AUFS) | Adjusted Peak Area $(cm^2)$ |
|---|---|---|---|---|---|
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**TEACHER'S GUIDE:**
**HOW MUCH ASPIRIN,... IS IN YOUR PAIN RELIEVER?**

CLASSROOM USAGE        Chemistry 2

CURRICULUM INTEGRATION

This lab would work well for second year students as an introduction to instrumental analysis. For this lab, students should have some understanding of polarity, solutions, solution concentration, and a mathematical understanding of standard curves.

PREPARATION

See **HPLC Instructions For Operation** handout.

TIME:  GETTING READY

Getting the HPLC up and running will take approximately 1 hour the first day.  On each succeeding day it will take approximately 20 minutes.

TIME: STUDENTS

Preparing samples will take about 5 minutes per sample.  Each run on the HPLC will last about 5 minutes.  Each standard curve will take approximately 30 minutes to run.  It is also possible to use copies of previous standard curve data to save time.  (See VARIATIONS)

SAFETY AND DISPOSAL

All solutions can be washed down the drain.

CALCULATIONS

Students may have trouble estimating an appropriate dilution for their unknowns to fit the solutions into the appropriate range of the standard curve.

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NOTE ON PROCEDURE

Due to lack of a pure ibuprofen standard and a good method for including ibuprofen in a successful separation, the lab has been designed not to include testing for ibuprofen.  Teachers are welcome to experiment with ibuprofen as a classroom project.
VARIATIONS

For easy variations, any one of the three compounds can be tested on an individual basis.  For instance, you could just do the standard curve for aspirin and then attempt to measure the amount of aspirin in your unknowns.

If running a standard curve would be too time consuming you could use the data below to create standard curves and focus student time on unknowns.

The following experimental data will introduce roughly 2-3% error into your results.  You will also notice that the range of concentrations is slightly different.  To compensate for the different ranges, the slope and intercept of the best fit line is included and can be used for the appropriate calculations.


**ASPIRIN**

| Concentration (mg/mL) | Adjusted Peak Area (Area x Attenuation) ($cm^2$) |
| --- | --- |
| 0.0096 | 0.336 |
| 0.00672 | 0.229 |
| 0.00384 | 0.135 |
| 0.00096 | 0.0437 |

Best Fit Line:    Adjusted Peak Area = 33.698 x (Concentration)  +  0.0081

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**CAFFEINE**

| Concentration (mg/mL) | Adjusted Peak Area (Area x Attenuation) $(cm^2)$ |
|---|---|
| 0.0022 | 0.409 |
| 0.00154 | 0.296 |
| 0.00088 | 0.171 |
| 0.00022 | 0.0445 |

Best Fit Line:   Adjusted Peak Area = 184.508 x (Concentration)  +  0.00668

**ACETAMINOPHEN**

| Concentration (mg/mL) | Adjusted Peak Area (Area x Attenuation) $(cm^2)$ |
|---|---|
| 0.00112 | 0.694 |
| 0.000784 | 0.428 |
| 0.000448 | 0.254 |
| 0.000112 | 0.0505 |

Best Fit Line:   Adjusted Peak Area = 626.488 x (Concentration)  -  0.0294