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HPLC Determination of Acetaminophen in an Analgesic

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Background

High Performance Liquid Chromatography (HPLC) has resulted from the application of gas chromatography instrumental techniques to classical liquid chromatography. In classical liquid chromatography (column chromatography), a solid material (stationary phase) is packed into a column and the mixture to be separated is introduced onto the top of the column. An eluting liquid (mobile phase) is washed through the column. If a component in the mixture is strongly attracted to the stationary phase, it will be retarded on the column and will be separated from components that are more weakly attracted to the stationary phase. There are four main mechanisms by which separation occurs. In adsorption chromatography, the sample components are selectively adsorbed onto the surface of the solid stationary phase. In liquid-liquid chromatography the stationary phase is a liquid coated onto or chemically bonded to a finely divided inert solid support. Separation occurs due to partitioning of the sample components between the mobile and the stationary phases. In normal phase chromatography the mobile phase is nonpolar and the stationary phase is polar. In reverse phase chromatography, the mobile phase is polar and the stationary phase is nonpolar. This experiment will utilize reverse phase chromatography with an octadecyl (C-18) column and a polar mobile phase consisting of methanol, acetonitrile, and water.

In ion exchange chromatography, the stationary phase is an ion exchange resin, and the separations that occur are due to the interactions of the sample component ions and the ion exchange sites on the resin. Elution is affected by increasing solvent strength of the mobile phase. In size exclusion chromatography, the stationary phase is a porous gel, and separation occurs on the basis of component size. The pore size of the gel can be selected within a narrow range. Large molecules are excluded from the pores and thus elute more quickly from the column.

Classical liquid chromatography is time consuming. Only a few samples can be analyzed on any one column before it must be re-packed. Complex separations are difficult. HPLC provides automation and greatly improves on the speed, resolution, and efficiency of column chromatography.

HPLC developed during the 1960s as the high-efficiency particles used to pack the column became progressively smaller. Stationary phases in use today are “microparticulate” column packings. They are uniform, porous silica particles with spherical shapes and 3-, 5-, or 10-μm diameters. Different chemical groups are bonded to the surface of the silica particles to produce bonded phases. The group bonded to the silica particles is selected depending on which of the four mechanisms of separation is to be employed. The most commonly used bonded phase has C-18 alkyl groups attached to the surface of the silica particles. These phases are called ODS (octadecylsilane) bonded phases.

The great versatility of HPLC lies in the fact that not only can the stationary phase in the column be varied, but also many diverse mobile phases and mobile phase compositions can be used. The solvent, or mobile phase, must be pumped through the column with a high-pressure pump, since the

small, packed particles resist solvent flow. Usually the column is 10–25 cm long with a 4- to 6-mm internal diameter. The columns are reusable for a very large number of samples. The entire system must be able to withstand the high pressure and must be chemically resistant to the mobile phase. Columns and the HPLC system are usually made of stainless steel or titanium. Small-bore columns (2 mm in diameter or less), which require less solvent but higher pressure, can also be used.

After eluting from the column, sample components are detected by an in-line detector. The most common HPLC detector is a UV-visible spectrometer, since most compounds have some absorption in the UV-visible region of the electromagnetic spectrum. Infrared, refractive index, and more recently, mass spectrometer detectors are also used. An electrical signal from the detector is displayed on a recorder or a computing integrator or with data capture software.

As with gas chromatography, the retention time for a solute is characteristic for the component under a particular set of conditions. However, since many different solutes have the same (or very close to the same) retention time under any one set of conditions, identifying a compound by retention time alone is not routinely possible. To obtain an unambiguous identification, specific structural detail is necessary. Some detectors can collect and store the UV-visible spectra of all of the components as they elute from the column. These spectra can be matched with those for standard compounds. A mass spectrometer detector allows the collection of mass spectra of each sample component. These spectra can be matched with libraries of recorded spectra for possible identification. Spectra from IR and NMR detectors can also be used to obtain definitive structural identification.

The basic components of an HPLC instrument are a solvent reservoir, a high-pressure pump, a column packed with high-efficiency stationary phase, an injector for introducing samples onto the column, an in-line detector, and a readout for the detector signal. All parts of the system that come in contact with the mobile phase must be made of materials, such as stainless steel, that are resistant to reaction with the mobile phase. All components on the high-pressure side of the system must be able to withstand the pressures involved. The “dead volume” (any empty space) must be kept to a minimum, since empty space will drastically reduce the efficiency of the system. Very small-bore tubing in short lengths is used to connect the various components, and the injector and detector are designed to have very small internal volumes.

The sample component signal from the detector appears as a peak on the readout. The area of the peak is proportional to the concentration of the sample component, and thus HPLC is used extensively for quantitative analysis. Peak area can be determined by an electronic integrator or with data acquisition software. (Older methods of peak area determination include triangulation and cutting and weighing the peaks.) Peak area is sensitive to operating conditions, and this creates some difficulties in using external standards to determine the concentration of an analyte. Many of these problems can be overcome by adding a known amount of an internal standard to the sample and standards and then measuring the peak area of the compound of interest relative to the internal standard peak area. It is expected that minor variations will affect the peak area of both the internal standard and the analyte by the same relative amount. The ratio of analyte peak area to internal standard peak area is plotted versus concentration of the analyte. The internal standard must be selected carefully. It must be a substance not present in the original sample for analysis, and it should be completely resolved from the analyte, yet elute close to the analyte. It must be of known purity. Note that many USP and other validated methods use external standards.

4-Acetaminophenol (4-AAP, common name acetaminophen) is used extensively as a nonprescription antipyretic and analgesic agent. Analysis of 4-AAP is important in quality assurance measurements in the pharmaceutical industry as well as in pharmacological studies on clinical toxicity. In this experiment 4-acetaminophenol will be extracted from an analgesic and quantitatively measured on an HPLC using 2-acetaminophenol (2-AAP) as an internal standard. Figure 1 shows the chemical structures of 4-acetaminophenol and 2-acetaminophenol.

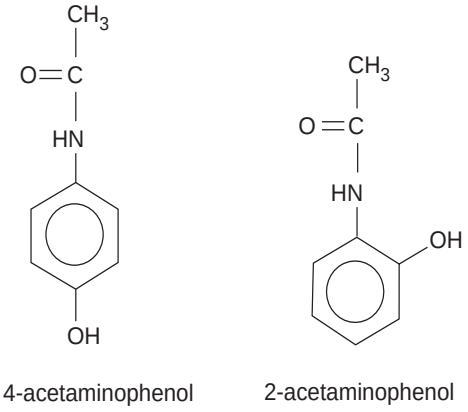

Figure 1: 4-Acetaminophenol and 2-acetaminophenol

Materials

- 2-acetaminophenol (2-AAP—Aldrich Chemical Company)
- 4-acetaminophenol (4-AAP—Aldrich)
- methanol, HPLC grade
- acetonitrile, HPLC grade
- deionized water
- millipore-type solvent filter fitted with 0.2-μm Nylon-66 filters
- analgesic containing 4-acetaminophenol (such as Extra Strength Tylenol® or Datril®)
- filter funnel fitted with Whatman #1 filter paper
- volumetric flasks
- round-bottomed culture tubes with screw caps fitted with PTFE liners
- micropipetters
- vortex evaporator
- HPLC with a UV detector set at 254 nm
- C-18 (ODS) column, 4.3 mm x 25 cm
- microliter Hamilton syringe
- mortar and pestle

Safety, Handling, and Disposal

It is your responsibility to specifically follow your institution's standard operating procedures (SOPs) and all local, state, and national guidelines on safe handling and storage of all chemicals and equipment you may use in this activity. This includes determining and using the appropriate personal protective equipment (e.g., goggles, gloves, apron). If you are at any time unsure about an SOP or other regulation, check with your instructor.

Preparation of Standards

1. Prepare stock solutions of 1 mg/mL 4-AAP and the internal standard 2-AAP by dissolving 100 mg of each compound in 100 mL methanol. Prepare a working solution of each.
   Since 15 μg of 2-AAP will be pipetted into each of the culture tubes, it is convenient to dilute the 2-AAP stock solution 10 mL to 100 mL (100 μg/mL) and then pipet 150 μL of the diluted solution into each tube. Varying amounts of 4-AAP ranging from 0.1 to 15 μg will be pipetted, so dilute the 4-AAP stock 1:100 (10 μg/mL).

2. Add 15 μg of the 2-AAP working solution to 18 round-bottomed culture tubes fitted with PTFE-lined screw caps. (Pipet 150 μL of the diluted stock.)

3. Evaporate the solvent to dryness at 50°C under mildly reduced pressure in a vortex evaporator.

4. To a series of 15 of the culture tubes prepare five standard concentrations (three replications of each) by adding varying amounts of 4-AAP ranging from 0.1–15 μg. (To set up a standard curve, 2, 4, 6, 8, and 10 μg of 4-AAP make five convenient standards. Pipet 200, 400, 600, 800, and 1,000 μL of the diluted 4-AAP stock, respectively, into the culture tubes containing the 2-AAP.)

5. Evaporate the organic solvent to dryness as before.

6. Carefully pipet 1.00 mL methanol into each culture tube and mix on a vortex mixer.

7. Filter all of the standards through a 0.2-μm Nylon-66 filter using syringe filters or centrifuge tube filters.

8. Store the standards at 4°C until HPLC analysis can be performed.

Preparation of Sample

1. Obtain one analgesic tablet such as Extra Strength Tylenol or Extra Strength Datril and crush thoroughly with a mortar and pestle.

2. Add 30 mL methanol to the mortar and mix well to extract the 4-AAP from the tablet. Allow the powder to settle, and quantitatively transfer the methanol extract through a filter funnel containing filter paper into a 100-mL volumetric flask. Repeat the extraction with two more aliquots of 30 mL methanol, making sure to quantitatively transfer each extract. Bring the final volume up to 100 mL with methanol.

3. Prepare a dilution of this extract and pipet a known volume into the three remaining culture tubes containing the internal standard.
   Use the stated amount of 4-AAP from the analgesic bottle to calculate a dilution that would fall within the range of the standards. Typically, the Extra Strength analgesic will contain 500 mg of 4-acetaminophenol. This means that the concentration of the extract will be 500 mg/100 mL total volume, or 5 μg/μL. Since the highest standard contains 10 μg 4-AAP, you will need to dilute the analgesic 1:100 and then pipet 100 μL of diluted sample into each of three culture tubes containing the internal standard, 2-AAP. The sample tube will thus contain 5 μg of 4-AAP, an amount in the middle range of the standards.

4. Evaporate the organic solvent to dryness and then pipet 1.00 mL methanol into each tube as before.

5. Filter the samples through a 0.2-μm Nylon-66 filter and store at 4°C until HPLC analysis.

HPLC Analysis

Preparation of mobile phase

1. The mobile phase for this analysis is acetonitrile:methanol:water (12:12:76). Solvents must be filtered to remove any particulate matter and degassed before use on an HPLC. If a pump system is available that can program the solvent mixture, filter each of the pure solvents—acetonitrile, methanol, and water—through a 0.2-μm Nylon-66 filter. Be careful to minimize exposure to dust particles. If the HPLC system is not capable of programming this solvent mixture, prepare 2 L of a 12:12:76 acetonitrile:methanol:water solution and then filter this mixture through a nylon filter.

2. There are several ways to degas the mobile phase. Vacuum filtration will remove dissolved air, but it must be performed before each use of the mobile phase. The mobile phase can be sonicated for 20 minutes prior to use to remove dissolved air. Another method of degassing is to use a helium sparge. Degas the mobile phase with the method conveniently available.

3. The HPLC column must be equilibrated with the mobile phase prior to analysis. Consult the manual provided with the HPLC to determine the best method for equilibration. The instructor will be able to provide assistance.
   Typically, an HPLC column is stored in a high concentration of methanol (70% methanol-water) to prevent microbial growth and corrosion of the metal surfaces. A 50-50 mixture of 70% methanol and mobile phase can be pumped through the column at a flow rate of 1.5–2.0 mL/min for about 20 minutes and then a mobile phase can be pumped at a flow rate of 2.0 mL/min for 20 minutes to equilibrate the column.

Analysis of samples

1. Set the flow rate on the HPLC at 2.0 mL/min. During the equilibration time begin setting up the data-handling device for use.

2. Consult with the instructor about the proper operation of an integrator or computer data capture software. The proper integration parameters for the analysis must be set. With an HP 3390A integrator, the following parameters work well:
   - area reject : 80,000
   - peak width: 0.16
   - threshold: 8
   - attenuation: 4

3. Zero the detector.

4. Inject 10 μL of each of the standards, beginning with the lowest concentration first. Inject all three replications of each standard concentration before moving on to the next. Carefully clean the microliter injection syringe. Inject 10 μL of each of the analgesic extract replications.

5. After all of the standards and samples have been injected, the column must be re-equilibrated for storage. Program the pump to deliver a 50-50 mixture of mobile phase and 70% methanol for 20 minutes at a flow of 1.5–2.0 mL/min. Alternately, prepare a 50-50 mixture of 70% methanol and mobile phase to pump onto the column. For the final equilibration, pump 70% methanol onto the column at a flow rate of 2.0 mL/min.
   Pumping the system through these equilibrations at the beginning and end of each day will greatly extend the life of the injector, column, and other components.

Results

The retention time for 4-AAP is about 2 minutes and for 2-AAP about 4.5 minutes under these conditions. (This can be verified by preparing appropriate standards for each of these compounds individually and injecting them on the HPLC.) Prepare a table of peak areas for 2-AAP and 4-AAP in each of the standards and in the analgesic sample. Calculate the ratio of 4-AAP to 2-AAP areas. Plot the average ratio for each amount of 4-AAP in the standards using a graphing program or linear graph paper. Determine the amount of 4-AAP in the analgesic from the graph. Calculate the total amount of 4-AAP extracted from the analgesic tablet.

Since the micrograms of 4-AAP determined from the graph was injected in 10 μL, then the concentration of the diluted extract will be:

X (from graph) μg/10 μL or X mg/10 mL

To find the total amount of 4-acetaminophenol extracted from the analgesic, multiply the concentration by the amount diluted (100 times) and then multiply by the total extraction volume (100 mL). When performed correctly, the milliliters will cancel and the total milligrams extracted will be calculated.

Compare the total measured to the amount stated on the bottle label.