Liquid Chromatography – Mass Spectrometry (LC/MS)

Prelab Questions: Questions to be answered before doing the experiment. **The answers are due at the beginning of each experiment without exception (the questions are for credit and some may appear on your final exam).**

1. Describe the LC-MS electrospray ionization (ESI) technology.
2. Why does the LC-MS tend to be the instrument of choice for many biological samples?
3. How is the Varian (Agilent) LC-MS 500 (MCAL lab) used in sample identification and quantitation?
4. What is meant by MS-MS, and what are the advantages of an instrument that has this capability?

Objectives:

1. Learn the principles and operation of LC-MS.
2. Develop methodology using MS and MS-MS in sample identification.
3. Become familiar with the concept of product ion fragmentation pathways.
4. Apply methodology to sample analysis.

Introduction:

The MS in MCAL uses electro-spray ionization (ESI) and Ion Trap technology to detect sample ions. The analyte ions are nebulized, dried in a stream of nitrogen and become charged ions. The ions can be scanned in full scale mode (MS), The ions can be further fragmented (MS-MS) and the product ions scanned.

The MS is connected to a binary LC system. Depending on the HPLC column used the LC can separate components in a mixture based on hydrophobicity, charge, and molecular size. A major difference between traditional HPLC and the chromatography used in LC-MS is that in the latter case the scale is usually much smaller, both with respect to the internal diameter of the column and even more so with respect to flow rate. The MS will give good sensitivity at flow rates of 200 μL/min or less. There are a lot of other mass analyzers besides Ion Trap that can be used in LC-MS; Single Quadrupole, Triple Quadrupole, TOF (Time of Flight) and Quadrupole-Time of Flight (Q-TOF).

Reagents and Standards:

1. Milli-Q water
2. C8 ,C12 or C18 RP column
3. HPLC-MS acetonitrile (Fisher gold), formic acid
4. Syringe filters
5. Analytical balance
6. Pipettes and disposable tips

7. Standards: Acetaminophen and caffeine. Analgesic medication of unknown concentration.

Optimization of MS conditions.

You will optimize the MS conditions for each of the analytes of interest. The three main parameters in full scan are the % RF and the needle and capillary voltage. Once you have obtained values for the precursor ion, you will optimize the CID (collision induced) voltage to determine optimal product ion conditions.

1. Standards of 1 mg/ml each acetaminophen, caffeine, are supplied to the students. Dilute the standards to 10 ug/ml in 5% Acetonitrile, 0.1% Formic Acid. Five mls of each is a good final volume. Filter the solutions through a 0.45 µm syringe filter.

2. Connect the syringe pump to the ESI source with the peek fitting. Rinse the syringe and then fill the MS syringe pump apparatus with 5% Acetonitrile and 0.1% FA.

3. Go to system control software and under the manual control tab and make sure the ion source, detector and RF are on. The ion source should always be on. Click on them if they are off. They will be green and show larger print when on. Purge for about 30 seconds by continually pressing the purge button. This will flow 200 µl/ min of the syringe solution into the MS. Monitor the baseline for a few minutes to determine background and ensure stability.

4. The MS should be set to *full scan* mode which is under the *active segment* tab. Set the range from 105 to 500 so the instrument is not producing a lot of excess data. Monitor the ions in the left hand bottom screen and look for the major ion(s). Positive mode generates the M+H ion (MW plus 1 H ion). If you are unable to observe the expected ion, it may be present as the M + Na (+23) ion or as a breakdown product ion.

5. Optimize needle voltage, capillary voltage and % RF loading in the MS on one standard at a time. This can be done under the *optimization plots* screen, inputting the molar mass of the ion to optimize in *plot these masses* window, and selecting the parameter to optimize in *dependant parameter* drag down. The MS will optimize each of needle voltage, capillary voltage and % RF as you select them and pressing *start* icon. Record the optimal values for each of your compounds.

6. Under the *active segment* tab set the optimization parameters (just determined) for one of the major ions of interest, then change the scan mode to MS/MS. Begin with the CID voltage at zero, you should see the parent ion in a similar intensity to scan mode. Now optimize the CID voltage (the energy used to dissociate the precursor ion) by clicking the optimization plots tab, and changing the dependent parameter to CID voltage.

7. Once CID voltage have been determined these can be set in the MS-MS mode window and product ion production monitored. This can be done for each ion of interest. Collect the spectral data of the precursor and product ions after optimization.

8. Repeat the MS-MS ^{3} and follow all product ions. Make a diagram of product ions breakdown (formation) and demonstrate in lab. write- up how they are connected (fractionated) to precursor ion. Try and identify as many of the major product ions (molecular formula) as you are able.

9. Collect all your data (your data should go into a folder as a descriptive name of run under 4590, 2016, Group?). Your chromatograms can be copied into a "Word" document and e-mailed to yourself and from "Word" copied into your write up.

Determination of unknown analgesic medication:

The identification of compounds in an analgesic solution can be determined either by injecting the sample through the syringe pump and determining the molecular weights of the various compounds in the mass spectrum, or by separating the analgesic on a HPLC column and looking at the separated compounds as they come out of the column and as their spectrum is displayed. In this experiment you will look at the spectrum of the mixture without separation and determine the concentration of the various constituents by comparing them to your previous standards as integrated area versus known concentration. This would basically be a one point calibration curve.

MS conditons:

Set the 500MS condition for optimum RF, needle, voltage, capillillary voltage and CID voltage conditions for the caffeine. Set the instrument to the MS-MS mode so that the caffeine will be broken down to produvt (daughter) ions. 6. Integrate a product ion of standards and sample (hold I key down and use cursor to draw baseline) to get area related to concentration. You can also integrate using the integration icon in the tool bar. Inject the analgesic solution and measure the integrated peak area of the major daughter ion. Run the analgesic mixture under the same conditions and measure the same ion's integrated area. Repeat tis for the acetaminophen and measure area of largest daughter ion. Compare these to previously ru individual samples of caffeine and acetaminophen and determine the concentration's of the two components in the unknown analgesic. All samples should be in 5% Acetonitrile, 0.1% Formica Acid.

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Data:

Identify each medication by retention time and product ions.
Identify the fragmentation patterns of the original and product ions and compare to literature patterns.
Determine concentration of medication.

Discussion:

Discuss your results in detail. Discuss the methodology, improvements that are possible. Discuss the fragmentation patterns and what is occurring using literature sources. Discuss concentration results, how it compares with label and reasons which might affect results, etc., etc.

Final Questions:

1. What are the reasons to use LC-MS versus GC-MS for the analysis of pharmaceutical and biological samples.
2. What are some of the problems associated with the use of this instrument for quantitative analysis? Compare advantages (disadvantages) of using full scan ions verses product ion from transitions for quantitation, and identification.
3. What parameters would you have to control to produce a library capable of identifying compounds based on their fragmentation pattern?
4. The instrument for this experiment is set to positive mode (can only see positively charged ions). What is the other routine mode the instrument can be set in and when would this mode be used and why?