Mass spectrometry for the chromatographer

“Mass spectrometry for the chromatographer” (60 minutes)
  LC/MS Basic Principles needed for peak tracking in method development
  Getting more seasoned with the MS lingo: TIC, XIC, SIM, MS and MS/MS.
  Ionization process (ESI). Formation of molecular ions, adduct ions, isotopes.
  MS compatible methods- mobile phases
  Effect of Instrumental parameters on MS response
  Effect of Experimental parameters on MS response
LC/MS in Drug Development

- Identify unknown peaks to support method development and validation
- Characterize degradation products in forced degradation studies and stability samples
- Elucidate drug degradation pathway to support excipient selection and formulation development
- Confirm peak identity for DS and related substances
- Support QA issues and NDA
- Support Manufacturing process and finished drug products
Features of LC/MS

Advantages:

• High sensitivity
• High selectivity
• Speed
• Combined power of separation and identification
• Molecular weight and structure information

Disadvantages:

• Some compounds are not ionizable
• Difficult to identify complete unknowns
• Limited structure information compared to NMR
Identification Strategy:
When you start LC/MS analysis....

- Is the method MS compatible?
- Conditions where degradation compounds form
- Synthetic scheme (Byproduct? Intermediate?)
- Structure of drug substance
  - active sites and functional groups
  - Number of nitrogen atoms, Isotope ratio
- Full MS spectrum of unknown: Which is [M+H]?
- MS/MS spectra of DS and unknown
- UV spectra of DS and unknown
LC/MS Analysis

Work Request

LC/MS compatible method

Confirm Chromatogram

Select Ionization Mode

ESI vs APCI, (+) vs (-)

Determine MW

MS/MS(n) Study

Proposed Structure

Report

No

Confirmation?

Yes

In-house Synthesis

LC/MS incompatible method

Modify HPLC conditions

Show Peak Equivalency

Isolation/Purification

NMR Confirmation
LC-MS

- **LC** – Separation of the mixture of analytes
- **Interface** – Separation of the analyte from the solvent
- **MA (mass analyzer)** – separation of the analyte molecular ion and fragments according to their mass to charge ratio
Infusion MS

- Sample in continuous flow
- No sample injection
- No LC separation
- MS spectrum obtained over a given time
- Improved S/N due to the average of multiple spectra
- Select LC flow to assist ionization
10-5 Torr


Formation of gas phase ions from solution phase
ELECTROSPRAY

Factors to consider

- Ionic strength
- Surface tension of the solvent
- Volatility of the solvent
- Character of the analyte ions in solution: solvated, ion paired, etc
- Mobile phase composition and amount of water
- pH of the mobile phase
Confirmation of molecular weight

ESI (+)  ESI (-)
1. Effect of instrumental parameters on ESI response

• Mobile phase flowrate*
• Capillary*/corona voltage
• Fragmentor*/cone voltage

All play important roles in analyte ions formation and ionization efficiency
Effect of flow rate on ESI response

Dependence of mass analyzed ion intensity of the BH\(^+\) ion of protonated cocaine (10\(^{-5}\)M) on flow rate.

Increasing the flow rate increases droplet size which decreases the yield of gas-phase ions from the charged droplets.
- Use shorter columns of same phase and proportionally decrease flow rate.

P. Kebarle, et.al, Anal. Chem. 1993, 65, 972A
Formation of small micron sized droplets is not a problem if flow rate, surface tension are low. An increase of these may make it difficult to for the electric field to produce the desired charged aerosol. The electric field strength can be increased to try to overcome those effects. However if electric field is too high will give rise to electrical discharge and this is detrimental to ES signal. Nebulizing gas can be used to help focus the # of ions transported into vacuum envelope of the mass spectrometer.

Effect of capillary voltage

G. Valaskovic, J. Murphy, M. Lee, Milestone development
Effect of Cone/Fragmentsor Voltage on fragmentation

As potential difference (deltaV) between end of capillary (N) and first skimmer (S) is increased the ions are accelerated through the background gas, which leads to more collisions.
Effect of experimental parameters on ESI response

A. Dependence of ion intensities on analyte concentration

Concentration effect of tetracycline on the signal intensity

A. Kamel, P.R. Brown, B. Munson, *Analytical Chemistry*, vol. 71, 968-977, 1999
B. Role of solution equilibria and solution-phase chemistry

Basic compound: \( pK_a = 6 \)

Acidic compound: \( pK_a = 3 \)

\[ pH = \text{analyte } pK_a, \text{ Analyte is 50\% ionized} \]

**Effect of Solution pH (ESI)**

- Variation of solution pH changes the acid-base equilibrium of an analyte species
- Alters the degree of positive/negative charging via protonation/deprotonation
- Should get increased intensity with decreasing pH for basic compounds in positive ion mode
- Should get increased intensity with increasing pH for acidic compounds in negative ion mode
- However ion-pairing, ion-association may lead to ion-suppression actually causing a decrease in ionization efficiency
- There is a critical point at which increase in protonation ceases to increase ionization efficiency for protonated bases.
- Effect of organic on the shift of ionization equilibria in the mobile phase.
Desired pH values were achieved using different combinations of 0.1% formic acid and 0.1% ammonium hydroxide at constant organic composition. Flow injection analysis

Effect of pH on \([\text{M-H}]^-\) negative-ion response of Ibuprofen.

Ibuprofen (206.28) (pKa = 4.41)

Effect of eluent pH

Full Scan ESI Efficiency of Antidepressants vs Concentration of Acetonitrile in the mobile phase

Mobile Phase: Aqueous//MeCN
Aqueous = 1 mMol Ammonium Formate, pH 4.32 with Formic Acid
Analyte Conc: 100 nM/mL
Infusion Rate: 20 uL/min
Effect of type of acidic modifier on electrospray response

- Analyte ion intensity affected by the presence and concentration of other electrolytes
  1) Depends on the propensity of ion-association which can lead to formation of neutral species which will lead to ion-suppression causing a decrease in ionization efficiency:

\[
\text{CCl}_3\text{COO}^{->}\text{CF}_3\text{COO}^{>>}\text{CH}_3\text{COO}^{~\text{Cl}^-} \quad \text{Mirza and Chiat, Anal.Chem. 1994, 66, 2898-2904)}
\]

2) May depend on volatility of acid (bp of acid) Propionic > Acetic > Formic > TFA
Conclusions

The electrospray ion intensity can be enhanced by:

• Concentration of analyte and instrumental parameters
• Solution phase pH
• Type and concentration of acidic modifier or volatile buffer
• Type and concentration of organic modifier
• Gas phase Reactions are important, feasibility depends on proton affinities of analyte versus modifier in eluent
• ESI is a complex process comprises of many different variables, no set of parameters will guarantee large signal intensity
MS Structure elucidation

Basic principle
- Nitrogen Rule
- Isotope ratio (Cl, Br, etc.)
- Adduct ions

LC/MS
- Post column addition (acid or base)
- MS/MS or CID: from fragments to substructures
- Hydrogen-Deuterium exchange
- Utilization of UV spectra to support MS identification

Other techniques for structure elucidation
- Exact MS: Elemental composition & chemical formula
- GC/MS: EI mass spectrum
- NMR or LC/NMR: Detail structure
Calculating Molecular Weight

Three ways to calculate molecular weight

**Average mass:**
Average atomic weight for each element, (Merck index, DS profile)
12.01115*60+1.00797*122+14.0067*20+…………..=1443.8857

**Monoisotopic (exact) mass:**
Exact mass of the most abundant isotope of each element
12.0000*60+1.0078*122+14.0031*20+……………..=1442.8796

**Nominal mass:**
Integer nominal mass of the most abundant isotope of each element
12*60+1*122+14*20+………………………………….=1442

Mass defect 1444:1443:1442. This is as good as being a mile off!

**ALWAYS use the monoisotopic mass when calculating the molecular weight in mass spectrometry!**
Nitrogen Rule

- Compounds with an odd number of nitrogen atoms have an odd molecular weight (1, 3, 5, 7…)
- Compounds with an even number of nitrogen atoms have an even molecular weight (0, 2, 4, 6…)

### Positive and Negative Adduct Ions

<table>
<thead>
<tr>
<th>Positive ion detection</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>[M+H]^+</td>
<td>M+1</td>
</tr>
<tr>
<td>[M+NH₄]^+</td>
<td>M+18</td>
</tr>
<tr>
<td>[M+Na]^+</td>
<td>M+23</td>
</tr>
<tr>
<td>[M+K]^+</td>
<td>M+39</td>
</tr>
<tr>
<td>[2M+H]^+</td>
<td>2M+1</td>
</tr>
<tr>
<td>[2M+NH₄]^+</td>
<td>2M+18</td>
</tr>
<tr>
<td>[2M+Na]^+</td>
<td>2M+23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Negative ion detection</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>[M-H]^−</td>
<td>M-1</td>
</tr>
<tr>
<td>[M-H+HOAC]^−</td>
<td>M+59</td>
</tr>
<tr>
<td>[M-H+TFA]^−</td>
<td>M+113</td>
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</tbody>
</table>

MeCN= +41  
MeOH= +32
Distinct molecular ion pattern can be used to determine the molecular weight.

ESI full scan mass spectrum of paclitaxel with mobile phase that contains 2mM ammonium acetate and acetonitrile. Kerns, et.al, 1994, American Chemical Society
## Relative Isotope Abundance of Common Elements

<table>
<thead>
<tr>
<th>ELEMENTS</th>
<th>ISOTOPE</th>
<th>RELATIVE ABUNDANCE</th>
<th>ISOTOPE</th>
<th>RELATIVE ABUNDANCE</th>
<th>ISOTOPE</th>
<th>RELATIVE ABUNDANCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>$^{12}\text{C}$</td>
<td>100</td>
<td>$^{13}\text{C}$</td>
<td>1.11</td>
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<tr>
<td>Hydrogen</td>
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<td>100</td>
<td>$^{2}\text{H}$</td>
<td>0.016</td>
<td></td>
<td></td>
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<tr>
<td>Nitrogen</td>
<td>$^{14}\text{N}$</td>
<td>100</td>
<td>$^{15}\text{N}$</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>$^{16}\text{O}$</td>
<td>100</td>
<td>$^{17}\text{O}$</td>
<td>0.04</td>
<td>$^{18}\text{O}$</td>
<td>0.20</td>
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<tr>
<td>Sulfur</td>
<td>$^{32}\text{S}$</td>
<td>100</td>
<td>$^{33}\text{S}$</td>
<td>0.78</td>
<td>$^{34}\text{S}$</td>
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<tr>
<td>Chlorine</td>
<td>$^{35}\text{Cl}$</td>
<td>100</td>
<td></td>
<td></td>
<td>$^{37}\text{Cl}$</td>
<td>32.5</td>
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<tr>
<td>Bromine</td>
<td>$^{79}\text{Br}$</td>
<td>100</td>
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<td></td>
<td>$^{81}\text{Br}$</td>
<td>98.0</td>
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</tbody>
</table>
Isotope ratios of Chlorine and Bromine

**Cl**

- M: 100
- M+2: 32.6

**Cl2**

- M: 100
- M+2: 65.3
- M+4: 10.6

**Br**

- M: 100
- M+2: 97.9

**Br2**

- M: 100
- M+2: 195
- M+4: 95.5
Basic Data Interpretation

Isotope information - some isotopic patterns are diagnostic of the presence of certain elements

Ratio of 3:1 = Cl

MS Spectrum of Glyburide

MWt = 509 thus odd number of N

M+1 = 25% thus approx 25 C atoms (actually 23)

M+2 / M ratio approx 3:1 thus Cl present

S increases M+2 intensity
Using LCMS to determine artifact peaks formed on-column

Acidic conditions
**TIC vs. EIC**

- **TIC**
- **m/z = 522, XIC**
- **m/z = 504, XIC**

The graphs illustrate the time (min.) vs. intensity for different mass-to-charge ratios (m/z) and LC-MS fragmentation methods. The top graph shows the total ion current (TIC) over time, with peak intensities at various time points.

The middle and bottom graphs display the extracted ion current (XIC) for specific m/z values, highlighting the intensity peaks at 522 and 504, respectively, over the same time frame.
Figure 3. LC/TOF/MS spectra and accurate measurements for the secondary amides of alachlor (a) and acetochlor (b) ethane sulfonic acids in a groundwater sample.

Post column addition

- Sample injection
- LC separation
- No impact on LC chromatographic pattern
- Improve ionization with either acid or base addition after LC column
- Select appropriate solution to assist ionization

MicroTee

1 ml/min → waste

1% Formic acid in IPA

0.1 ml/min → MS
Nitrogen Rule

This rule is derived from the fact that, perhaps coincidentally, for the most common chemical elements in neutral organic compounds (hydrogen, carbon, nitrogen, oxygen, silicon, phosphorus, sulfur, and the halogens), elements with even numbered nominal masses form even numbers of covalent bonds, while elements with odd numbered nominal masses form odd numbers of covalent bonds, with the exception of nitrogen, which has a nominal (or integer) mass of 14, but has a valency of 3.

It should be noted that the nitrogen rule is only true for neutral structures in which all of the atoms in the molecule have a number of covalent bonds equal to their standard valency (counting each sigma bond and pi bond as a separate covalent bond for the purposes of the calculation). Therefore, the rule is typically only applied to the molecular ion signal in the mass spectrum.

Mass spectrometry generally operates by measuring the mass of ions. If the measured ion is generated by creating or breaking a single covalent bond (such as protonating an amine to form an ammonium center or removing a hydride from a molecule to leave a positively charged ion) then the nitrogen rule becomes reversed (odd numbered masses indicate even numbers of nitrogens and vice versa). However, for each consecutive covalent bond that is broken or formed, the nitrogen rule again reverses.

Therefore, a more rigorous definition of the nitrogen rule for organic compounds containing exclusively hydrogen, carbon, nitrogen, oxygen, silicon, phosphorus, sulfur, and the halogens would be as follows:

An even nominal mass indicates that a net even number of covalent bonds have been broken or formed and an even number of nitrogen atoms are present, or that a net odd number of covalent bonds have been broken or formed and an odd number of nitrogen atoms are present. An odd nominal mass indicates that a net even number of covalent bonds have been broken or formed and an odd number of nitrogen atoms are present, or that a net odd number of covalent bonds have been broken or formed and an even number of nitrogen atoms are present.