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Current Tools for Quantitative LC-MS in the Clinical Laboratory

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Best Practices and Current Applications for
Mass Spectrometry in the Clinical Laboratory
September 17, 2013

Learning Objectives

After this presentation, you should be able to:

1. Explain why we still need liquid chromatography when using mass spectrometry
2. List the components of HPLC and UPLC systems and the attributes of each type of LC
3. Explain the differences between HPLC and UPLC
4. List the different mass spectrometers available and compare the data acquisition capabilities
5. Evaluate which mass spectrometer would best suit the applications required in your laboratory

Overview

- Liquid chromatography
  - overview of LC components
  - HPLC and UHPLC
  - comparison
- Mass analyzers
  - triple quadrupole, single quadrupole, ion trap
  - SIM, SRM, ion ratios and product ion spectra for confirmation
  - high resolution mass analyzers and data acquisition
  - nominal mass vs exact mass
  - comparison of mass analyzers
- What's still needed
- Conclusions

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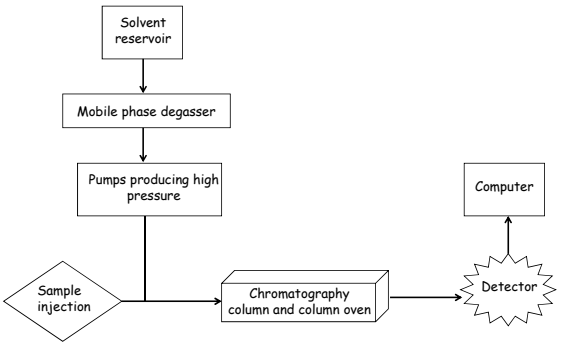
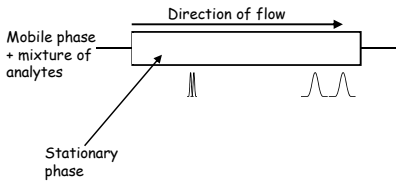
Liquid chromatography components



What is liquid chromatography?

A technique used to separate a mixture of analytes in a sample that is dependent upon how each analyte interacts between a stationary phase (the chromatography column) and a mobile phase (solvent mixture flowing through the column)



Why do we still need liquid chromatography when using mass spectrometry?

- to separate analytes of interest from matrix components
- to separate analytes of interest from interfering substances
- to separate isomeric or isobaric analytes before MS or MS/MS analysis
  - e.g. testosterone and epitestosterone
  - morphine and hydromorphone
- to aid in the ionization process
- retention time can be used as part of the identification criteria of analyte of interest

Modes of chromatography commonly used with MS

Reverse phase chromatography:

Stationary phase is more non-polar (hydrophobic) than the mobile phase (e.g., C18, C8, phenyl columns)

Non-polar analytes are retained for longer by the column

Most commonly used mode

Normal phase chromatography:

Stationary phase is more polar (hydrophilic) than the mobile phase (e.g., hydrophilic interaction chromatography (HILIC), amine, cyano)

Polar analytes are retained for longer by the column.

Mobile Phase Composition

Usually consist of:

Water
Organic solvent (e.g., methanol, acetonitrile, hexane)
Additives (e.g., formic acid, ammonium hydroxide, ammonium acetate/formate/bicarbonate)

NOTE: always use the highest quality water, solvents and additives available!!

Reverse phase chromatography example:

Mobile phase A: 0.1% formic acid in water

Mobile phase B: 0.1% formic acid in 70:30 methanol:acetonitrile

Normal phase chromatography example:

Mobile phase A: 10 mM ammonium formate, 0.125% formic acid in 90:10 acetonitrile:water

Mobile phase B: 10 mM ammonium formation, 0.125% formic acid in 50:50 acetonitrile:water

Isocratic vs Gradient Elution

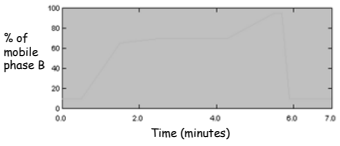
Isocratic elution:

Same mobile phase composition throughout the whole run

Gradient elution:

Mobile phase composition (and therefore strength) changes during the run – most commonly used in LC-MS applications

Time (minutes)	% A	% B
0.0	90	10
0.5	90	10
1.5	35	65
2.5	30	70
4.3	30	70
5.5	5	95
5.7	5	95
5.9	90	10
7.0	90	10



Isocratic vs Gradient Elution

Isocratic elution

Advantages

Run to run retention time stability

Highly reproducible peak areas

Challenges

Increased peak width with later retention times

Gradient elution

Advantages

Increased analyte selectivity

Removal of matrix interference

Challenges

Requires higher complexity pumps

Sensitive to minor variations in mobile phase mixing

Increased variation in retention time and peak area

Requires longer equilibration between samples

Types of Liquid Chromatography

High performance liquid chromatography: HPLC

Pumps deliver maximum pressure of ~ 400 bar/6000 psi

Plastic polymer tubing is mostly adequate

Ultra high performance liquid chromatography: UHPLC

Pumps deliver maximum pressure of ~ 1200 bar/18000 psi

Stainless steel tubing is required

How does stationary phase particle size determine LC pump pressure?

History of LC column particle size:

Initially: 40 µm

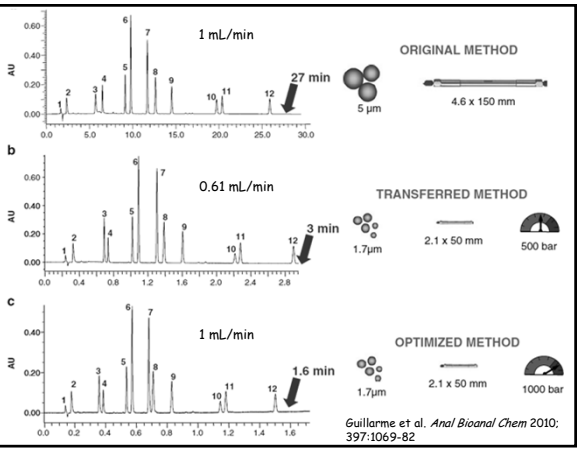
1970s: 10 µm

1990s: 2 µm

Currently: < 2 µm

The smaller the particle size of the chromatography column:

- more surface area available
- better separation (resolution)
- higher back pressure generated in LC pumps



Guillarme et al. *Anal Bioanal Chem* 2010; 397:1069-82

What happens if the pressure in your system exceeds the maximum pressure?

- LC system will shut down no matter what it is doing at that time
  - i.e., your run will stop and you may lose precious samples!
- you will hear an annoying beeping sound
- there could be damage to the column or fittings in the LC system

NOTE:

- pressure will change during run if using gradient elution
- methanol causes higher pressure than acetonitrile
- increased column oven temperature reduces pressure

Factors to consider when choosing HPLC vs UHPLC

- difficulty involved in the separation of analytes in a complex mixture
- daily testing volume
- new method development
- cost

HPLC: less maintenance; no high pressure pumps, valves, mixers, tubing and fittings; fused-core columns allow use of smaller particle size at lower pressures

UHPLC: small sample volumes; consumes less solvent; higher sample throughput. But due to high pressure, requires more maintenance, more expensive fittings, column lifetimes are shorter and therefore method can be more expensive

HPLC vs UHPLC implementation considerations

Staffing

- some technologists in clinical laboratories will already have HPLC experience, but may not have UHPLC experience
- using UHPLC requires a better understanding of the effects of mobile phase composition and sample extraction

Equipment cost

- HPLC = ~ $40,000-$90,000 (depending on configuration/manufacturer)
- UHPLC = ~$70,000 - $110,000 (depending on configuration/manufacturer)
- plus service contract – significant cost!

HPLC vs UHPLC

HPLC

Advantages

Can use any LC equipment

Vast array of columns chemistries/vendors available

Fused-core columns allow for higher resolution

Can be more rugged

Challenges

To obtain increased resolution requires longer run times

UHPLC

Advantages

Increased resolution

Increased speed of analysis and therefore throughput

Less solvent per sample

Less sample volume

Challenges

Requires dedicated equipment

Dead volume critical

Sample preparation and mobile phases are more critical

Can require more maintenance

Column chemistries/vendors limited

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Mass Spectrometry

- when coupled to HPLC or UHPLC, mass spectrometers function as an ion detector
- need to ensure mass analyzer can acquire data fast enough for high throughput, high resolution UHPLC methods especially if quantifying multiple analytes

Types of mass analyzers:

single quadrupole

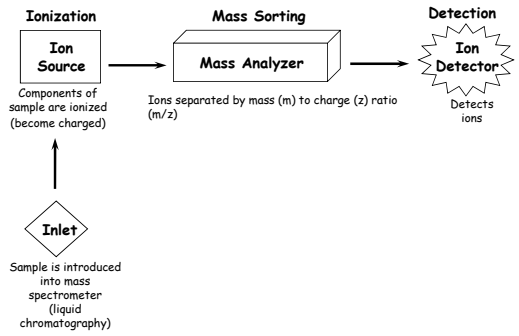
triple quadrupole

quadrupole ion trap

time of flight

Fourier transform ion cyclotron resonance (FTICR)

What are the components of a mass spectrometry system?



Other considerations for implementing mass spectrometry

- electrical supply
- gas supply – nitrogen, argon
- exhaust
- UPS or back up power
- roughing pump and oil (and disposing of oil)

Mass spectrometry vendor should be able to give you a site guide documenting the requirements for the instrument

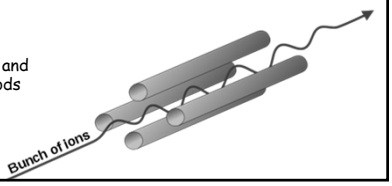
Optional - interface between mass spectrometer and laboratory information system

These can all add $$$ to the cost of implementation!

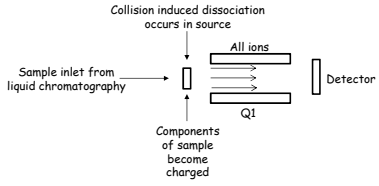
What is a quadrupole?

- four metal rods set parallel to each other
- each opposing rod pair is connected electrically and a radio frequency (RF) voltage is applied between the rod pairs
- direct current voltage is superimposed on the RF voltage
- only ions with a certain mass to charge ratio will move through the quadrupole at the specific voltages
  - allowing one m/z to be monitored or to scan for a range of m/z by varying the voltages

Other ions will have unstable trajectories and will collide with the rods

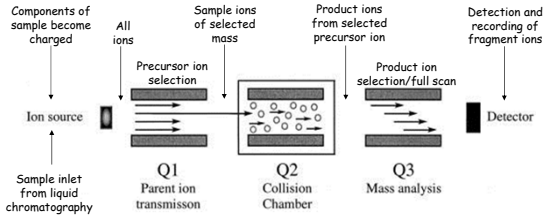


Single Quadrupole MS



- only ions of desired mass to charge ratio reach detector when using optimized voltages for analyte of interest
- can also scan across a mass range by varying voltages
- all analytes with that mass will be detected
- not as specific as other instruments

Triple Quadrupole MS



Q1. Ions of interest are selected (precursor/parent ions)
Q2. Fragmented into smaller product ions
Q3. Product ions separated by mass (m) to charge (z) ratio (m/z)

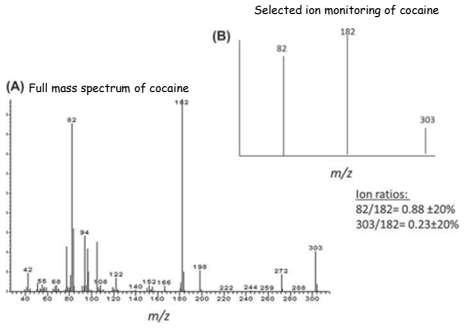
- also known as a tandem mass spectrometer (MS/MS)
- very selective so best for quantitative analysis
- poor scanning capabilities

Quadrupole Ion Trap MS

- quadrupole used to generate a field that functions to "trap" ions without destroying them
- ideal for qualitative analysis and elucidation of ion structure
- not as useful for quantitative analysis due to capacity limitations of the trap
- can be used to produce product ion spectra if used with MS/MS
  - an extra layer of selectivity

What are the commonly used modes of operation using these instruments?

Selected Ion Monitoring (SIM)

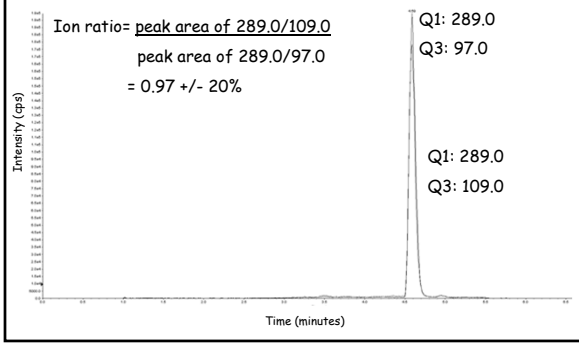


Wu AHB, et al. Clin Toxicol 2012;50(8):733-42

Selected Ion Monitoring (SIM)

- typically employed in clinical laboratories using GC- or LC-MS
- targeted method
- monitoring fragmentation pattern of specific ions
- usually monitor 3 ions (may include molecular ion and fragment ions)
- use ratios between relative abundance of ions to ensure specificity
- ion ratios consistent across calibrators, controls and patient samples
- improves sensitivity, selectivity and precision of method

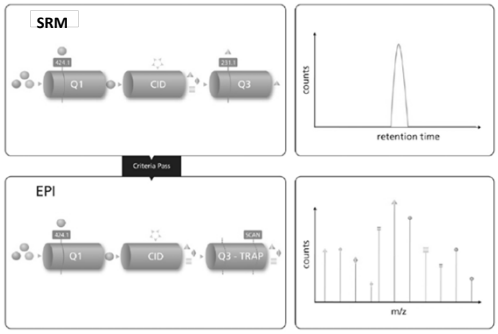
Selected Reaction Monitoring (SRM)



Selected Reaction Monitoring (SRM)

- typically employed in clinical laboratories using LC-MS/MS
- targeted method
- monitoring of precursor/product ion pairs – transition
- usually monitor 2 transitions per analyte and internal standard
- use ratio between 2 transitions to help determine if there are interferences in the LC-MS/MS method – ion ratios
- ion ratios consistent across calibrators, controls and patient samples
- improves sensitivity, selectivity and precision of method

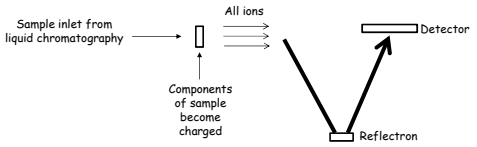
SRM and product ion spectra



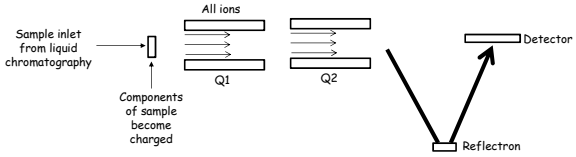
Courtesy of AB SCIEX

High resolution mass analyzers

Time of flight MS (TOF-MS)



Quadrupole time of flight MS (QTOF-MS)

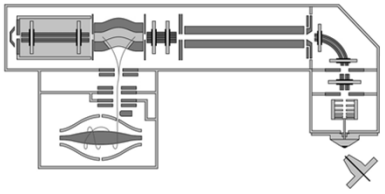


Time of flight MS (TOF-MS)

- based on time it takes for an ion to travel a specific path length when the same force is applied to all ions
- lighter ions arrive at detector earlier than heavy ions
- theoretically TOF-MS has no m/z range limit
- linear dynamic range limitations due to detector saturation
- useful for accurate mass determination
- not as useful for quantitative analysis unless using QTOF-MS

Fourier transform ion cyclotron resonance MS

- FTICR-MS (Orbitrap technology uses similar principles)



- ions trapped in a cell inside a strong magnetic field and move in circular orbits in a plane perpendicular to magnetic field
- RF electrical potential is applied to transmitter plates causing trapped ions to be excited into larger circular orbits
- frequency of motion of ion is inversely proportional to its mass

What defines a mass analyzer as "high resolution"?

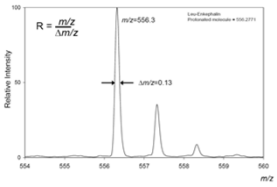
Mass Resolution

The ability to distinguish between ions differing slightly in m/z ratio

Can be calculated in two different ways:

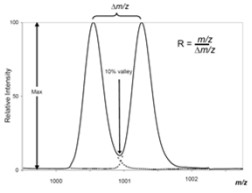
Δm/z is the full width of the peak at half its maximum height (FWHM).

Resolution = 556.3/0.13 = 4279



m/z of lowest mass peak is divided by the difference in m/z of the peaks (Δm/z).

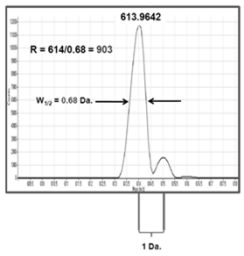
Resolution = 1000/1= 1000



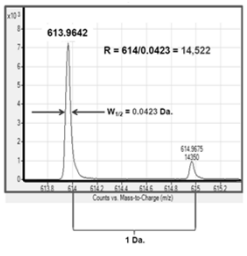
CLSI C50-A document

Mass Resolution

Single and Triple Quad, Ion Trap



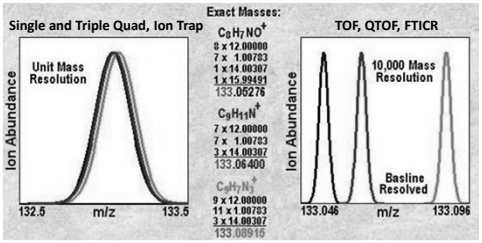
TOF, QTOF, FTICR



Courtesy of Agilent Technologies

Nominal Mass vs Exact Mass

- nominal mass = mass of an molecule calculated using atomic masses of each atom taken as integers
- exact mass = calculated mass based on adding up the masses of each atom in a molecule



http://www.epa.gov/esd/chemistry/ice/faq.htm#wiaem

What are the commonly used different modes of operation using these instruments?

TOF-MS

- full scan of all ions in sample
- extract chromatogram to obtain accurate mass
- database search to identify compound as well as matching LC retention time

QTOF-MS

- full scan of all ions in sample and set criteria to trigger MS/MS
- extract chromatogram to obtain accurate mass
- database/library search to identify compound based on fragmentation pattern, accurate mass, ion ratios, LC retention time

FTICR

- full scan and full scan fragmentation of all ions in sample
- extract chromatogram to obtain accurate mass
- database/library search to identify compound based on accurate mass, fragmentation pattern and LC retention time

Which instrument do you need?

	LC-MS	LC-MS/MS	LC-TOF-MS	LC-QTOF-MS	FTICR
Specificity	++	+++	++	+++	+++
Sensitivity	++	+++	++	+++	+++
Resolution	Low	Low	High	High	Highest
Mass Accuracy	~0.1 units	~0.1 units	~0.01 units	~0.01 units	~0.0001 units
Ease of Use	++	+++	+++	++++	++++
Suited for which Applications?	Targeted Quant	Targeted Quant	Untargeted Qual	Targeted or untargeted Quant	Targeted or untargeted Quant
Cost	$$	$$$-$$$$	$$	$$$$	$$$$$

DO NOT FORGET THE COST OF A SERVICE CONTRACT – SIGNIFICANT $$$

Factors to consider when choosing instrumentation

- what do you actually need for the applications you wish to implement?
  - take into account sensitivity, throughput, and robustness requirements for your lab
- what expertise do your technologists possess?
- what is the cost – direct and indirect – of implementation?

What is still needed in terms of LC and MS?

- automation of the whole process
- ready to use reagent kits
- more user friendly software
- autoverification of results
- easier implementation of an interface between the MS and the laboratory information system
- service available 24/7
- reduction in cost ☺

Conclusions

- HPLC has wide applicability in clinical laboratories using easily available instrumentation and a wide variety of column chemistries
- UHPLC increases throughput while reducing solvent cost but initial investment is larger, maintenance and consumables are more expensive and currently there are not as many column chemistries available
- mass analyzers vary in specificity, sensitivity, cost and ease of use – they should be chosen wisely in terms of desired applications
- don't forget the "extras" such as gas and electrical supply, exhaust, service contract, etc., as the cost is significant