LC-MS/MS in the Clinical Laboratory

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LC-MS/MS in the clinical laboratory

• LC-MS/MS is mainly used for quantification of analytes used in the diagnosis and monitoring of disease
• Semi-quantitative assays are used for e.g. drugs of abuse analysis
• Screening for unknown compounds e.g. legal highs
• MALDI-ToF fingerprinting in microbiology.
Clinical Biochemistry at UHSM

- Serve population of 570,000
- 2,160,000 tests per year
- Main bulk of work carried out on automated analysers
LC-MS/MS at UHSM

- 6 mass spectrometers
  - 1 x Waters Quattro Premier
LC-MS/MS at UHSM

• 6 mass spectrometers
  – 2 x Xevo TQD
LC-MS/MS at UHSM

• 6 mass spectrometers
  – 1 x Xevo TQ-MS
LC-MS/MS at UHSM

• 6 mass spectrometers
  – 2 x Xevo TQ-S with Online Sample Manager
LC-MS/MS at UHSM

- 25 routine analyses by LC-MS/MS
- Therapeutic drug monitoring
  - Immunosuppressants
  - Antifungal
  - Prednisolone
- Tumour markers
  - Metanephrines
  - 5-HIAA
- Steroids
  - Cortisol
  - Aldosterone and renin
  - Androgens
- Urine tests
  - Urine stone screens
  - Cotinine
Steroids (MRM)

- Vitamin D
- Cortisol (serum, urine & saliva)
- Testosterone (serum & saliva)
- Androstenedione
- DHEAS
- 17-hydroxyprogesterone
- 11-deoxycortisol
- Cortisone
- Aldosterone
- Oestradiol
- Progesterone.......etc
- Synthetics e.g. prednisolone......etc
Other clinical Uses

- Paediatrics
- Drugs of abuse/toxicology
- Haemoglobin
Ion suppression

• Ion suppression is a matrix effect that causes a decrease in ionisation of the analyte of interest.

• This can vary between patient samples, so it is important to identify ion suppression in an assay, to remove it if possible or to compensate for it.
Causes of ion suppression

• Co-elution with analyte of interest of:
  – Non-volatile components e.g. salts
  – Endogenous compounds e.g. peptides, amino acids, phospholipids
  – Plasticisers
  – Drugs/metabolites
  – Drug delivery vehicles e.g. PEG
  – Ion pair reagents
Causes of ion suppression

In general:

– Molecules of higher mass tend to suppress ionisation of those of lower mass
– More polar analytes are susceptible to suppression
Mechanism of ion suppression in ESI

• No-one really knows!
• Theories include:
  – Competition for charge between analyte and suppressor
  – Large concentrations of non-volatile agents affecting the surface tension of the droplet & affecting evaporation efficiency
Detection of ion suppression

• Post column infusion of compound into MS whilst injecting extracted sample via HPLC.
• If endogenous analyte, may need to infuse stable isotope of analyte instead
• It is important to carry out ion suppression experiments using plasma from different sources AND collected in all the types of tubes you are likely to encounter.
Detection of ion suppression
Detection of ion suppression
Quantification of ion suppression

- Prepare 3 types of standard curves using 5-6 lots of plasma from different sources:
  - 1 in mobile phase
    - baseline recovery
    - Also gives an idea of system reproducibility
  - 1 in plasma which has been spiked post extraction
    - This shows any matrix effects
  - 1 in plasma spiked pre-extraction
    - Adds in the effects of extraction efficiency

Matuszewski et al, Anal. Chem. 2003: 75; 3019
Quantification of ion suppression

• Ideally, all 3 curves should overlay:
Quantification of ion suppression

• In real life, this may not occur.
• To calculate ion suppression due to matrix effects:
  \[(\text{Peak area of analyte spiked after extraction/peak area of analyte spiked into mobile phase}) \times 100\]
• \(<100\% = \text{ion suppression}\)
• \(>100\% = \text{ion enhancement}\)
Quantification of ion suppression

• To calculate the extraction efficiency:
  \[
  \frac{\text{Peak area of analyte spiked before extraction}}{\text{Peak area of analyte spiked after extraction}} \times 100
  \]

• To calculate the efficiency of the whole process:
  \[
  \frac{\text{Matrix effect (\%)} \times \text{extraction efficiency (\%)}}{100}
  \]
Quantification of ion suppression

- Mean peak area in mobile phase = 10000
- Mean peak area spiked pre-extraction = 7500
- Mean peak area spiked post-extraction = 9000
- Matrix effect = $\frac{9000}{10000} \times 100 = 90\%$
- Extraction efficiency = $\frac{7500}{9000} \times 100 = 83\%$
- Process efficiency = $90 \times 83 / 100 = 75\%$
Quantification of ion suppression

• +/- 10% is probably acceptable.
• Ion suppression/enhancement greater than this may require changes in the method.
Internal standards

• The same amount of internal standard is added to each standard, sample and QC.

• Using the response of sample/internal standard compensates for changes in sample extraction, ionisation efficiency and some ion suppression.
Internal standards

• Ideally we use a stable isotope labelled version of the analyte of interest.

• $^{13}\text{C}$ or $^{15}\text{N}$ versions are best as there is a greater difference in physico-chemical properties between H isotopes than the others due to the greater size difference

• $^2\text{H}$ binds more strongly to carbon than $^1\text{H}$ which may introduce small differences in chromatographic separation between analytes and deuterium labelled internal standards, especially if UPLC is used, reducing their ability to compensate for ion suppression effects
Stable isotope internal standards

1. $^2$H$_{11}$-amphetamine
2. $^2$H$_3$-amphetamine
3. $^{13}$C$_6$-amphetamine
4. Amphetamine
5. $^2$H$_{11}$-methamphetamine
6. $^2$H$_5$-methamphetamine
7. $^{13}$C$_6$-methamphetamine
8. Methamphetamine
Sample preparation

• The method of sample preparation is very important

• Choice of sample prep can depend on:
  – Sample type
  – Concentration of analyte of interest
  – Interfering substances
  – Ion suppression
  – Equipment availability
Sample dilution

- Simplest method of sample preparation
- Most suited to aqueous samples where matrix effects won’t be an issue
- Can be used for urine samples or saliva, but ion suppression may be a problem
- Examples – urine 5-HIAA
Protein precipitation

• Zinc sulphate is used to precipitate large proteins such as immunoglobulins. In a whole blood assay, it also lyses the cells to release the contents.

• Acetonitrile or methanol is then added to precipitate smaller proteins and also zinc sulphate.

• Samples are centrifuged before analysis and only the supernatant is injected.

• Examples of this include cyclosporin and tacrolimus, sirolimus and everolimus, MPA and voriconazole.
Liquid liquid extraction

- Separates compounds based on their relative solubilities in two immiscible liquids.
- Solvents commonly used include hexane, DCM and MTBE.
- Solvent is added to sample plus internal standard, then mixed to extract.
- The solvent layer is separated from the matrix, and can be dried down to add a concentration step.
Liquid liquid extraction

Organic solvent

Sample

Mix

Separate
Liquid liquid extraction
Liquid liquid extraction
Supported liquid extraction

• Uses modified diatomaceous earth as a solid support for liquid liquid extraction.
• Sample is diluted with water and internal standard, then applied to the support.
• After adsorption onto the diatomaceous earth, the analyte is eluted, then the solvent evaporated and sample reconstituted.
Supported liquid extraction
Solid phase extraction

• This uses the affinity of solutes dissolved in the liquid mobile phase for the stationary solid phase
Solid phase extraction

- Condition
- Equilibrate
- Load sample
- Wash
- Elute
Solid phase extraction
Solid phase extraction
Solid phase extraction
Solid phase extraction
Solid phase extraction
Solid phase extraction
Isobaric interferences

- Isobaric refers to another molecule with the same mass
- These may also fragment to give same daughter ions
Isobaric interferences

• 21 deoxycortisol, 11 deoxycortisol & corticosterone
Isobaric interferences

- Natural isotope of any carbon containing compound
  - $M + 1$, $M + 2$

- $M + 2$ will have same $m/z$ as doubly deuterated internal standard
Isobaric interferences

Duxbury et al, Ann Clin Biochem 2008
Isobaric interferences

Cortisol (nmol/L) vs. Response

- 15 ug/L d2-cortisol
- 3.75 ug/L d2-cortisol
- 1.5 ug/L d2-cortisol

R² = 0.9954

Cortisol (nmol/L) vs. Response
Isobaric interferences

Reported M+2 effect of prednisolone in cortisol assays

Cortisol $\text{Mr} = 362$

Prednisolone $\text{Mr} = 360$
Isobaric interferences

• Chromatography required to separate isobaric interference
• Can be difficult due similar structure
• Particular problem for steroids
  – Structural isomers
  – M+2 isotopes
Quantifier and qualifier transitions

- Two transitions tuned per compound
- Most sensitive transition used for quantification
- Secondary transition used for qualification
- Ion ratios monitored
  - Should remain constant
- Difference in ion ratio suggests interference
Calibrators

• Some kits aimed at LC-MS/MS available
• Often include columns, precipitation reagent and internal standards (and calibrators)
• Most assays use calibrators prepared in house
• Can be the source of wide inter-laboratory differences
• Anchor to reference material or comparison with reference laboratory
  – Often not possible or very expensive
• Comparison with established LC-MS/MS assays
Calibrator matrix

• Serum/plasma
  – Simple for drug assays
  – Difficult for e.g. steroid assays
• Animal serum e.g. horse
  – Not the same as human matrix
• Charcoal stripped serum/plasma
  – Adulterated
• Aqueous based e.g. PBS based
  – Only if no matrix effects in assay
Conclusion

• The main use of mass spectrometry in clinical labs is for quantification of analytes
• Internal standards are used to compensate for ion suppression and for changes in extraction and ionisation efficiency
• Many different sample preparation techniques are available, and the choice of technique depends on analyte concentration, ion suppression, interfering analytes and equipment availability
• Automation is making sample preparation much faster and more robust.
• There are many different things to take into account when developing an assay, such as isobaric interferences, choice of calibrator and the plastics used in sample preparation.