Taking Aim at Interference
(Without Shooting Yourself in the Foot)

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Introduction

- Popularity of LC-MS/MS-based methods for clinical testing continues to increase
- One of the major reasons: superior analytical specificity
- Despite that, these methods may still suffer from interference
  - affecting method accuracy and precision
  - negatively impacting patient care

Aim of this presentation

Introduce the participant to:

- Sources of guidelines for interference testing in method development/validation and routine testing
- What is analytical interference and where does it come from?
- How do we define acceptable interference levels?
- How do we test for interference in LC-MS/MS?
- When do we test for interference?
- The use of internal standard in mitigating interference
- How do we monitor for interference?

Guidelines for interference testing

- CLSI – Clinical and Laboratory Standards Institute
  - EP7-A2: Interference testing in clinical chemistry
  - EP14-A2: Evaluation of matrix effects
  - C62-A: Liquid chromatography-mass spectrometry methods
- FDA – Food and Drug Administration
  - Guidance for Industry. Bioanalytical Method Validation
- SWGTOX – Scientific Working Group for Forensic Toxicology
  - SWGTOX Doc 003: Standard Practices for Method Validation in Forensic Toxicology
- WADA – World Anti-Doping Agency
  - WADA Technical Document – TD2015IDCR: Minimum criteria for LC-MS confirmation of the identity of analytes for doping control purposes
- European Medicines Agency
  - Guidelines on bioanalytical method validation

What is analytical interference?

- Interference = the effect of a substance, identified or not, that causes the measured concentration of an analyte to differ from its true value.

  (Reference: Evaluation of matrix effects; Approved guideline – 2nd edition. CLSI document EP14-A2. Wayne (PA): CLSI; 2005.)

- Interferent or Interfering Substance = the substance causing interference

What is analytical interference?

- Interference
  - may appear in an assay as partially or completely co-eluting peaks in the analyte or internal standard mass chromatograms
  - may be virtually invisible to the naked eye – a matrix effect
    - caused by interfering substance altering the efficiency of the analyte and/or internal standard ions reaching the MS detector.

Where does interference come from?

- Interfering substances
  - may come from many different sources
  - may be introduced at any time before or during the testing workflow

Examples of interfering substances

- Compounds related to patient treatment
  - drugs
  - parenteral nutrition
  - plasma expanders
- Metabolites produced in pathological conditions
- Substances ingested by patients
  - alcohol
  - drugs of abuse
  - nutritional supplements
  - food
- Substances added during sample preparation
  - anticoagulants
  - preservatives
  - stabilizers
- Contamination during sample handling
  - hand lotion
  - serum separators
  - collection tube stoppers
  - leachables from plastic consumables
- Interferences arising from the sample matrix
  - hemolysis, icterus, lipemia.

How do we define acceptable interference levels?

- Acceptability criteria must be decided prior to conducting evaluation to ensure objectivity
- Key question:

  How large a discrepancy is considered clinically significant?
- Accuracy requirements:
  - Have been proposed for some analytes (total allowable error)
  - Can be established based on physiological variability
  - Can be derived from clinical experience (consensus of clinical experts)
  - Can be based on analytical variability (long-term imprecision)
- For more detail:

  CLSI document EP7-A2. Interference testing in clinical chemistry; Approved guideline – 2nd edition. Wayne (PA): CLSI; 2005.

Why do we test for interference?

- Interference affects:
  - method accuracy
  - method precision
  - quality and validity of reported results
- Assessing susceptibility to analytical interference – a very important part of any LC-MS/MS method development and validation

How do we test for interference in LC-MS/MS?

Interference testing falls into two categories:

1) Direct testing of the effect of specific substances on analyte concentration
2) Evaluation of unidentified interferences arising from sample matrix and anything added to it

Testing for specific interference

- Pool patient specimens containing analyte of interest
- From the pool generate:
  - Test samples – spiked with potential interferent
  - Control samples – spike with solvent matching the solvent of potential interferent
- Analyze both test and control samples in the same manner as patient specimens
  - with adequate replication
  - within one analytical run
- Evaluate interference as bias of the target analyte concentration in test vs control sample
- Initially, test substances spiked at the highest concentration expected in patient specimens
- When substances produce a clinically significant interference, they should be evaluated further at different concentrations to determine the magnitude of the interference

Testing for specific interference

- **Advantages**
  - Ability to define acceptable/unacceptable sample collection conditions and abnormalities
  - Ability to provide guidelines for patient preparation
    (medications, supplements, and foods to avoid prior to sample collection)
    - To obtain a valid test result
    - Reduce a need for repeat specimen collection and analysis
- **Disadvantages**
  - Laboriousness of testing a large number of substances
  - No practical interference study can identify all potential interferents

Testing for unidentified interference

- LC-MS/MS allows testing for interference that cannot be anticipated or identified beforehand
- Interference arising from sample matrix (matrix effects) can cause
  - Signal enhancement
  - Signal suppression
- Evaluation of matrix effects
  - Quantitative matrix effect study
  - Qualitative post-column infusion study

Quantitative matrix effect study

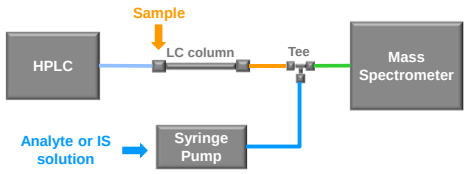
- Analyte added to:
  - extracted test samples (typically blank matrix)
  - control samples (typically solvent based, no matrix elements)
- Test and control samples analyzed in the same manner as patient samples
- The signal of test sample expressed at % of control sample signal:
  - <100% indicates suppression
  - >100% indicates enhancement

Quantitative matrix effect study

- The extent of a matrix effect can be calculated as:
  - non-normalized (as a ratio of peak areas) or
  - normalized to internal standard (as a ratio of response factors, which are analyte peak areas divided by internal standard peak areas).
- Both matrix effect values provide valuable information:
  - non-normalized – actual magnitude of ion suppression/enhancement
  - normalized values – how well the IS compensates for the matrix effect
- These experiments should be performed:
  - At two concentrations expected in the patient population
  - With several native matrix sources, such as different patient specimens or different vendor sources.
- Useful when testing matrices used for calibrator or QC preparation

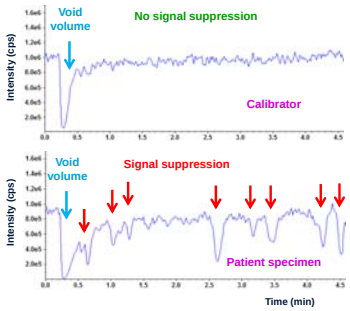
Qualitative post-column infusion study

- Analyte solution infused into the LC column effluent while analyzing blank sample matrix
- When blank sample matrix not available/not representative of patient specimens, a solution of IS may be infused while analyzing a patient specimen



Qualitative post-column infusion study

- Ion suppression/enhancement evaluated as the presence of negative/positive peaks in a steady signal trace of the infused analyte or IS
- **Advantage**
  - Allows for visualization of the position and width of matrix effects regions
  - Useful in optimizing separation conditions



When do we test for interference?

- Interference testing often performed as part of **method validation**
- Waiting until method validation to perform these experiments can result in unwanted surprises
- To ensure a developed LC-MS/MS method is robust and provides high quality data, test for interferences:
  - as part of the **method development** process
  - by performing the experiments outlined
- **Post-column infusion study** very useful for:
  - designing an LC gradient that will maneuver analytes out of suppression zones (especially in the case of dilute-and-shoot methods prone to matrix effects)
  - assessing extract cleanliness when determining
    - which sample preparation method or
    - which conditions may best mitigate matrix effects
- Note: Interference testing and the adjustment of method parameters may need to be an iterative process
- Labs should use as many patient specimens as practical to ensure that they capture the biological variability of interference

Don't shoot yourself in the foot!

Investigate interference EARLY on!

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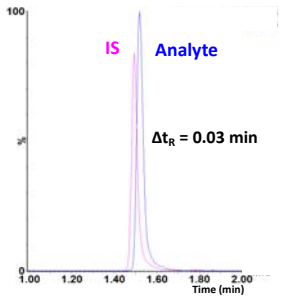
Interference mitigation by internal standard

- Many ways to reduce interference, but
- No method is completely immune to interference
- Use of stable isotope-labeled internal standards (IS) to mitigate interference (such as signal suppression) common
- When analyte elutes in a suppression region, compensating with an IS often deemed adequate
- **Problems:**
  1. Separation effect: IS not exactly co-eluting with the analyte
     => Differential suppression
     => Assay accuracy compromised
  2. Severe suppression by matrix: Coeluting IS not compensating for matrix effects
     => Analyte and IS S/N ratio drastically reduced
     => Assay performance compromised, especially near LLOQ

Problem #1: Separation effect



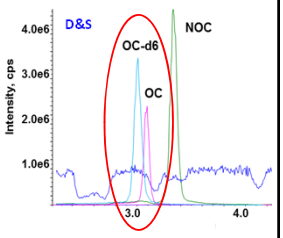
- Deuterated analogs mostly affected
  - esp. when deuteriums in positions impacting chromatographic retention
- Differences in physicochemical properties

  => differences in interaction with mobile & stationary phases

  => differences in retention times
- Degree of separation
  - Molecule size
  - Number of D labels
  - Position of D labels
  - LC conditions

Differential suppression by sample matrix

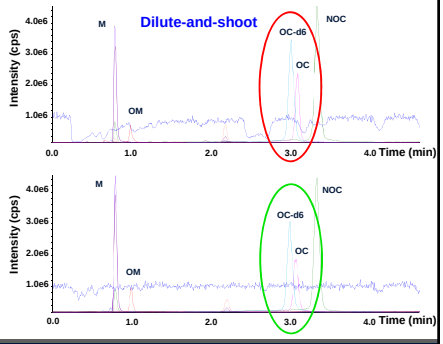
- Analyte and IS suppressed by matrix to different degrees
- Quantitation errors
  - Over-quantitating: if IS more suppressed
  - Under-quantitating: if analyte more suppressed
- The larger the separation, the lower the IS ability to compensate for matrix effects



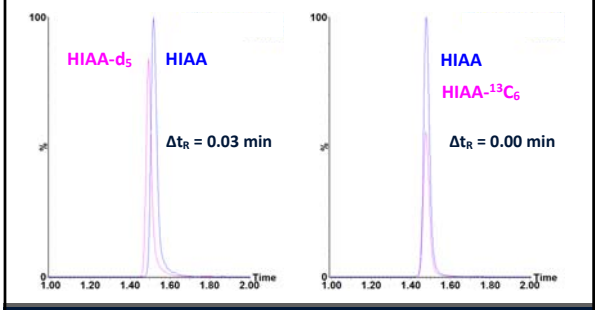
Solution A:

Scenario:
- Differential suppression between analyte and IS

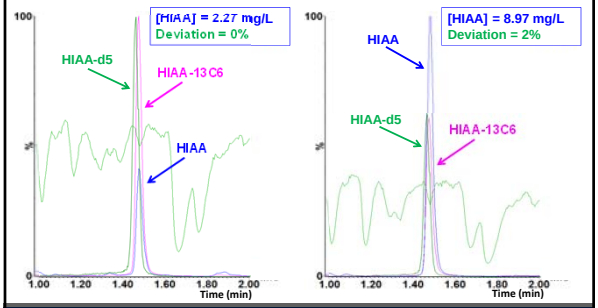
Solution:



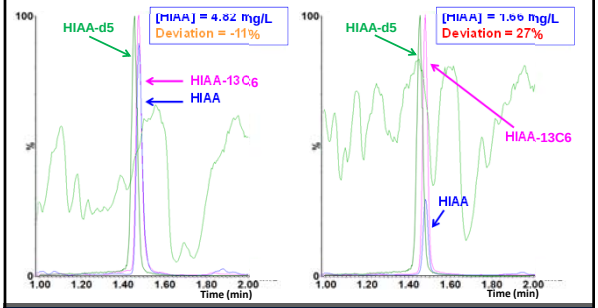
Solution B:



Post-column infusion experiment: samples with < ±10% deviation



Post-column infusion experiment: samples with > ±10% deviation



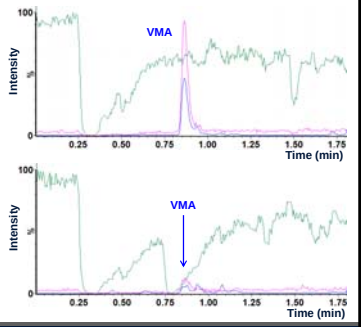
Alternate isotope labels: ^{13}C,^{15}N,…

Advantages	Drawbacks
➢ Do not suffer from label instability	➢ More difficult to make
➢ Closer co-elution with analyte	➢ More expensive
➢ Better compensation for matrix effects	➢ Not always commercially available

Problem #2:
Coeluting IS not compensating for matrix effects

Normal scenario:
Very little to no signal suppression by matrix components

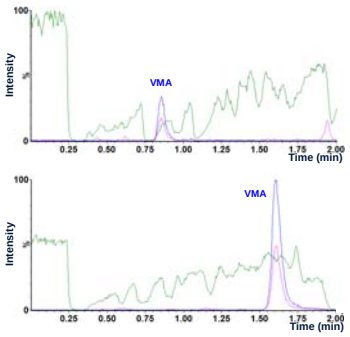
Severe matrix effect:
Signal suppression severely decreases S/N for analyte and IS
=> Assay performance compromised, especially near LLOQ



Solution C:

Scenario:
Suppression zone partially coeluting with analyte in primary validated method with faster gradient

Solution:



Mitigation by internal standard???

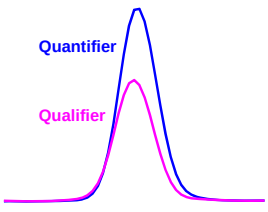
- **Problems:**
  1. Separation effect: IS not exactly co-eluting with the analyte
     => Differential suppression
     => Assay accuracy compromised
  2. Severe suppression by matrix: Coeluting IS not compensating for matrix effects
     => Analyte and IS S/N ratio drastically reduced
     => Assay performance compromised, especially near LLOQ
- **Solutions**

How do we monitor for interference?

- Even the best method development strategies rarely able to prevent interference completely
- Need to monitor for interference in routine testing in order to avoid reporting compromised results
- Data quality metrics
  - Ion ratios
    - Interferents isobaric with analyte/IS
    - Appear on analyte/IS transitions
  - Absolute IS areas
    - Interferents cause signal suppression
  - Retention times
    - Near-eluting isobaric interferent integrated instead of analyte/IS

Ion ratios –
Monitoring for isobaric interferents in chromatograms

$$Ion\ ratio = \frac{Quantifier\ Mass\ Transition\ Peak\ area}{Qualifier\ Mass\ Transition\ Peak\ area}$$

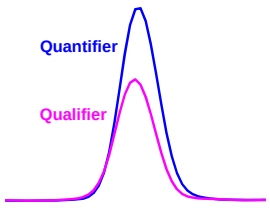


Ion ratios –
Monitoring for isobaric interferents in chromatograms

- Ideally calculated for both analyte and internal standard
- Mean ion ratio – calculated from ion ratios of calibration standards/ quality controls
- Individual specimen ion ratios compared to mean ion ratio
- Acceptance limits set during method development
  - based on clinical requirements for the assay
  - ±20 or 30% common

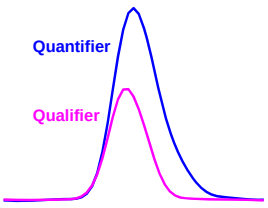
Ion ratios –
Monitoring for isobaric interferents in chromatograms

No interference



Quantifier peak area	1.02E+05
Qualifier peak area	6.43E+04
Ion ratio	1.58
IR±20% range	1.26-1.89

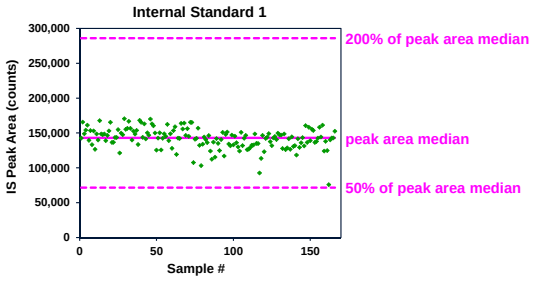
Interference



Quantifier peak area	1.18E+05
Qualifier peak area	5.57E+04
Ion ratio	2.11

Absolute IS areas –
Monitoring for signal suppression

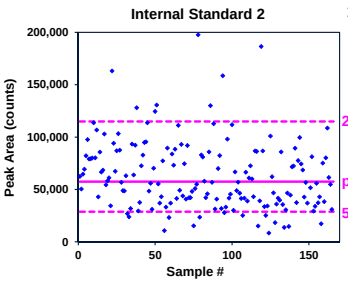
Well-controlled assay



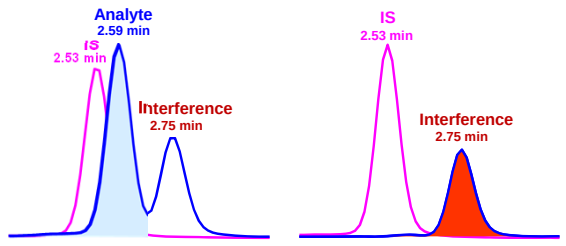
Absolute IS areas –
Monitoring for signal suppression

Not so well-controlled assay

- Typical of dilute&shoot methods – large variations due to signal suppression
- Can indicate samples extraction issues – variable recovery



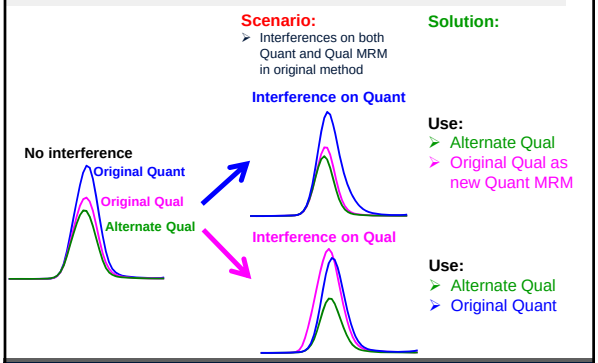
Retention times (absolute or relative to IS) –
Monitoring for near-eluting isobaric interferent integrated in place of analyte/IS



Metanephrine interference

Scenario:
- Interferences on both Quant and Qual MRM in original method

Solution:



Use:
- Alternate Qual
- Original Qual as new Quant MRM

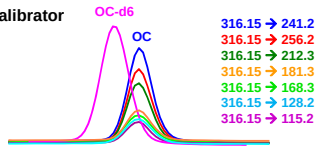
Use:
- Alternate Qual
- Original Quant

Coeluting Oxycodone interference

Scenario:

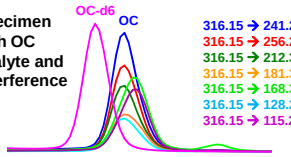
- Unresolved Oxycodone interference in original method
- Added all viable MRMs
- All MRMs give signal for both analyte and interference

Calibrator



316.15 → 241.2
316.15 → 256.2
316.15 → 212.3
316.15 → 181.3
316.15 → 168.3
316.15 → 128.2
316.15 → 115.2

Specimen with OC analyte and interference



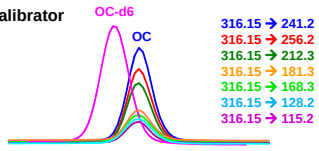
316.15 → 241.2
316.15 → 256.2
316.15 → 212.3
316.15 → 181.3
316.15 → 168.3
316.15 → 128.2
316.15 → 115.2

Coeluting Oxycodone interference

Scenario:

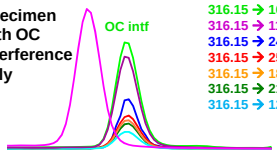
- Unresolved Oxycodone interference in original method
- Added all viable MRMs
- All MRMs give signal for both analyte and interference
- But in different ratios!!!

Calibrator



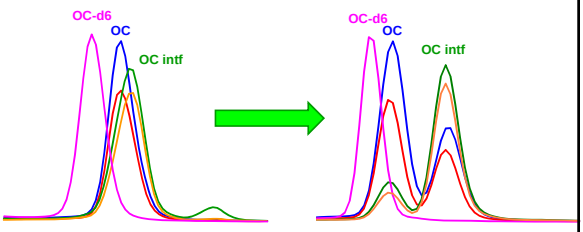
316.15 → 241.2
316.15 → 256.2
316.15 → 212.3
316.15 → 181.3
316.15 → 168.3
316.15 → 128.2
316.15 → 115.2

Specimen with OC interference only



316.15 → 168.3
316.15 → 115.2
316.15 → 241.2
316.15 → 256.2
316.15 → 181.3
316.15 → 212.3
316.15 → 128.2

Coeluting Oxycodone interference



Solution:

References:

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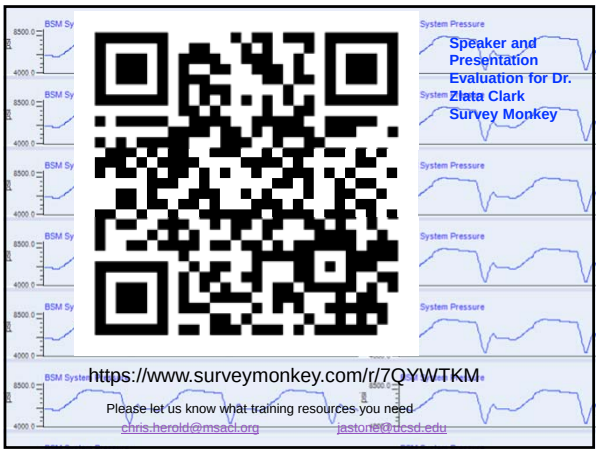
Questions?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