CLSI C60: Method Development

Julianne Cook Botelho, Ph.D.

Clinical Chemistry Branch/Division of Laboratory Science

CDC

AACC Conference –Mass Spectrometry in the Clinical Lab: Best Practice and Current Applications

Chicago, IL

September 6, 2012

Disclosures

- Nothing to disclose
- Any mention or allusion to specific vendors or vendor parameters should not be seen as endorsement

Outline

- Pre-analytical Considerations
- Internal Standard (IS) and Calibrator
- Assay Development/Optimization
- Matrix Effect Evaluation and Pre Validation

Pre-Analytical Considerations

- **Analyte of Interest- what type of compound are you looking to analyze?**

  - **<u>Exogenous Compounds</u>**- NOT produced *in vivo*
    - therapeutic drug monitoring or toxicology
      - tacrolimus, cocaine

  - **<u>Endogenous Compounds</u>**- produced *in vivo*
    - lipids, steroids, protein biomarkers
      - testosterone, cortisol, metanephrines

Common Considerations:

Specimen Type, Collection, and Pathways

Pre-Analytical Considerations

- **Exogenous Compounds**
  - Specimen type
    - **whole blood, serum, plasma urine**; saliva, oral fluids, hair
  - Specimen collection
    - Timing- pre-dose, post-dose, multiple time points
  - Compound Pathway
    - Determine measurement of exogenous substance or metabolites
      - Understand the metabolism of exogenous compound
      - Understand the pharmacokinetics of the metabolites

Pre-Analytical Considerations

Endogenous Compounds

- Specimen type
  - **whole blood, serum, plasma urine**; saliva, oral fluids, hair
- Specimen collection
  - Timing- time of day, fasting, menstrual cycle, stage of maturation
  - Gender
  - Supine, Standing, Posture
- Compound Pathway
  - Understand the biological pathway of the compound.

Pre-Analytical Considerations

- **Common Concerns- Endogenous and Exogenous**
  - Bound and Unbound small molecules
    - total, free, bioavailable i.e. testosterone and SHBG
    - conditions could alter protein binding i.e. pregnancy, malnutrition, dehydration
  - Lipids- interfere with ionization
    - ID the influences of lipid/lipoprotien concentrations on analysis
    - Test extremes during validation
  - Isotopomers- same precursor mass that share a fragment mass with the analyte of interest

    .

Calibrators
Selection, Preparation, and Use

Not to be confused with calibration of the mass spectrometer

- **Selection**
  - Highest available purity
    - COA (certificate of analysis) and verify in house
  - **Certified Reference Standards are preferred**
    - When not available validation of calibrators outlined in CLSI CX05-R (*Metrological traceability and its implementation; A Report*)
  - Consider hygroscopic nature of the compounds- avoid adsorption of water, heat- assisted drying might be needed

Calibrators
Selection, Preparation, and Use

- **Preparation**
  - Gravimetrically weigh material- even first few stock solutions if possible to improve accuracy
  - Minimize serial dilutions to avoid additive pipette errors
  - Insure material dissolves completely in solution, keeping in mind the solubility of the material
  - Temperatures should be controlled during preparation with a water bath and calibrated pipettes and glassware should be used
  - If organics are used (methanol/ethanol) extreme caution taken with evaporation of stock solutions

Calibrators
Selection, Preparation, and Use

- **Preparation (con't)**
  - Prepare at concentrations representing the analytical range with 6-8 non-zero calibrators
  - Prepared in similar matrix used in the analysis
    - Exogenous Compounds- **drug free human fluids** collected in a similar manner to test samples
    - Endogenous Compounds- uses **a 'proxy' matrix** with compound removed by dialysis or stripping of native material or use of synthetic material
    - If matrix match is not possible **solvent** based (methanol) when method is free from matrix effects
    - Slope equivalency should be demonstrated (can be part of matrix evaluation)

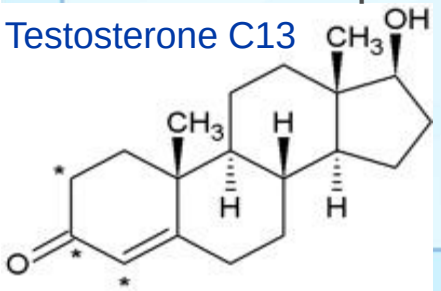
Internal Standard (IS)

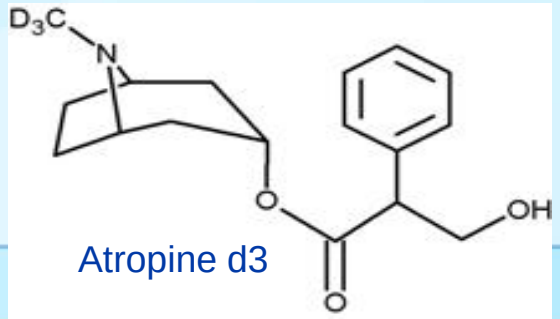
- **The “ID” is ID LC-MS/MS- isotope dilution**
- **Physiochemical Mimic of Analyte being measured**
- **Introduced prior to sample analysis**
- **Used to account for recovery variance**
  - Sample extraction
  - LC separation variance
  - Ionization Effects

Internal Standard (IS) Selection, Preparation, and Use

- **IS Selection**
  - Stable labeled isotopically enriched form of the analyte
    - Deuterium (^{2}H or D), Carbon-13 (^{13}C), Nitrogen-15 (^{15}N), and Oxygen-18 (^{18}O)
  - Similar physiochemical properties and retention time to analyte
  - Does not exhibit co-eluting inferences
  - Does not overlap the isotopic envelope of the analyte
    - Molecules <1000amu, at a minimum **+3** isotope enrichment is preferred

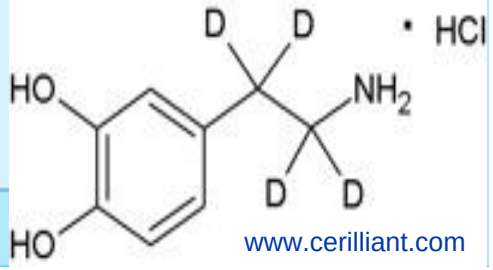
Testosterone C13





Atropine d3

Dopamine d4 +HCl



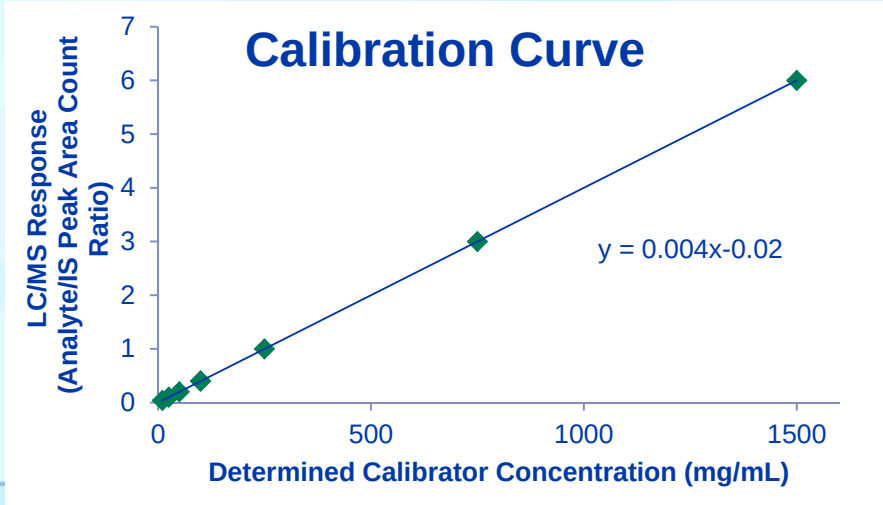
Internal Standard (IS) Selection, Preparation, and Use

- **IS Preparation**
  - Prepared at a **SINGLE** concentration at a reproducibly measureable level- recommended about approx 10-50x LLOQ
  - Insure selected concentration does not interfere with LLOQ via intrinsic impurities or contribution of unlabeled compound
  - Prepare with similar caution as calibrators
    - Gravimetric approach preferred
    - Avoid excessive serial dilutions
    - Insure solubility of material
    - Control temperature
    - Avoid evaporation

Calibrators and IS Selection, Preparation, and Use

Calibrator and IS Use

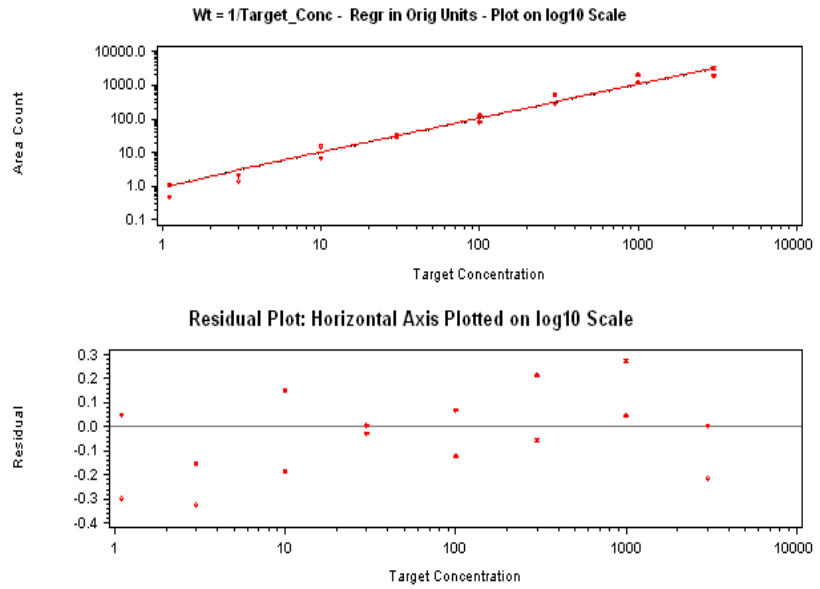
- IS is added at a single concentration to all calibrators *(QCs and Samples)*
- Calibrators are analyzed at the beginning of each batch
- Instrument response of the peak area count of analyte and IS are compared as a ratio to the determined calibrator concentration
- Simple approach used 1st degree polynomial y=mx+b

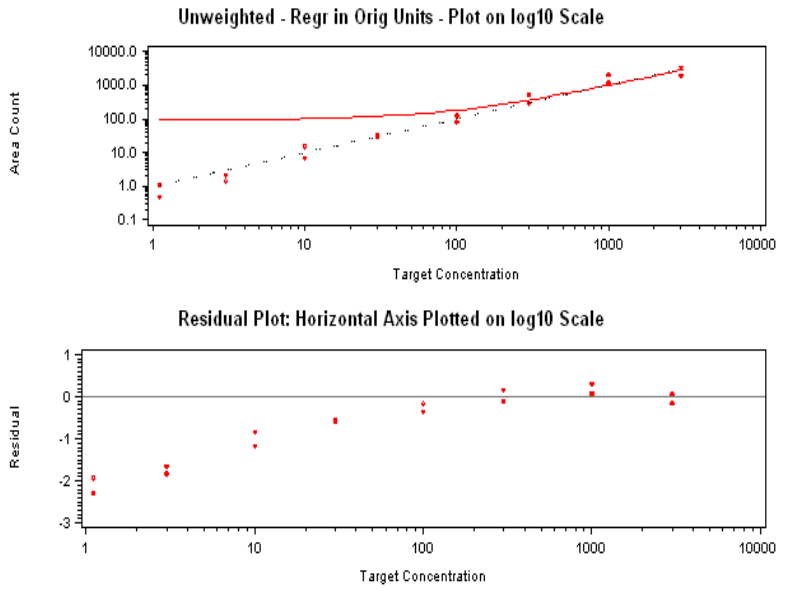


Calibrators and IS Selection, Preparation, and Use

Calibrator and IS Use (con't)

- Curve fitting can be considered using **weighted least square**
- Each approach should be thoroughly evaluated and determine which approach shows lowest sum of squared residuals and an even distribution in both the positive and negative direction for all calibrators.





Method Development

Start Neat- *if you can't get it here, matrix will be worse!*

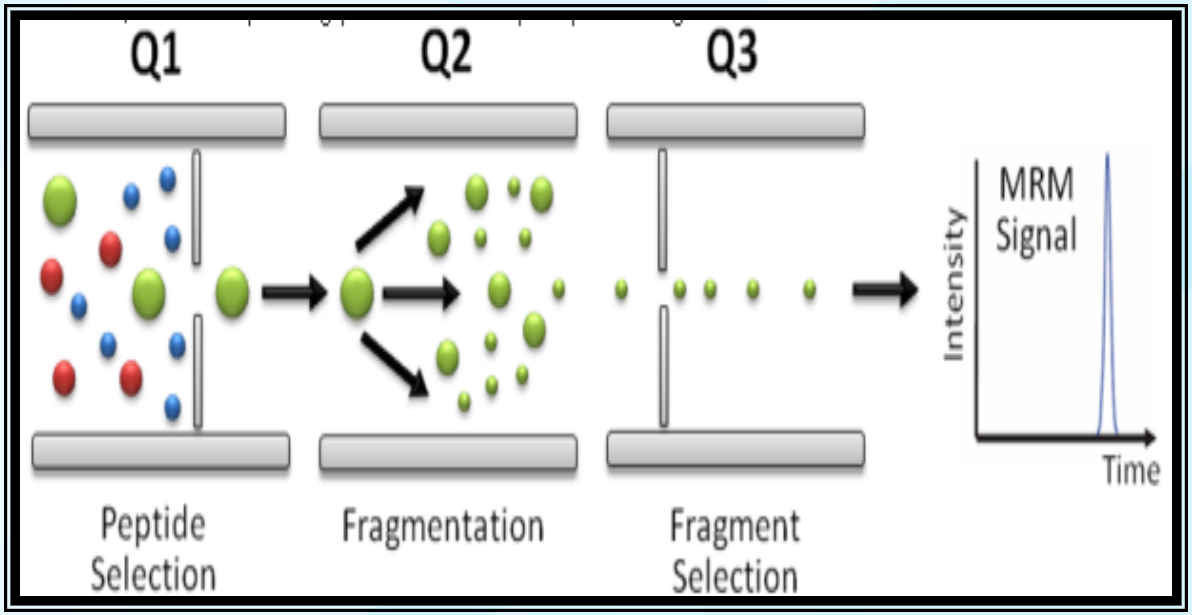
- MS- select transitions/optimize response
- LC- determine retention time/optimize peak shape
- LC/MS- optimize analyte ionization

Then move to **Matrix**

- LC/MS- optimize peak resolution
- Assess matrix effects
- Preliminary Evaluation

Method Development- Mass Spectrometer

- **Selective Reaction Monitoring (SRM)** or Multiple Reaction Monitoring (MRM)



Detector acts as a counting device for ions that match selected transitions- providing an intensity value over time

Method Development- Mass Spectrometer Transitions

- Specific pair of m/z values associated with the precursor (Q1) and the product ions (Q3) selected
  - Ie Testosterone 289>97

- Selecting transitions-
  - Precursor Ions gives highest/most stable signal
    - Check for adducts ie NH_4^+, Na+, K^+
  - For Product/Fragment Ions
    - Avoid loss of less than 18 mass units (ie loss of water)
    - Avoid loss of ammonia, carbon monoxide and carbon dioxide
    - If derivatization used in sample preparation avoid monitoring loss of derivative

- Final Method- 2 Transitions are recommended for Analyte and IS
  - Qualifier Ion
  - Confirmation Ion

Assay Development- Mass Spectrometer

Compound MS Optimization – via direct infusion of pure analyte (and IS) at a high concentration* in 50% methanol or acetonitrile

- **Instrument Polarity**: Positive vs. Negative Ionization
  - Can hypothesize with functional groups of analyte
    - Acidic compounds (negative) and basic compounds (positive)
  - Investigate mobile phase additives (more later)

- **Parameters**
  - Evaluate declustering potential (DP) and entrance potential (EP) to determine which produces the optimal signal for the precursor ion (analyte and IS)
  - Determine optimal collision energy (CE) and exit potential (CXP) which produces optimal signal for each product ion

** high enough but not too high to avoid contamination of interface and saturation of detector*

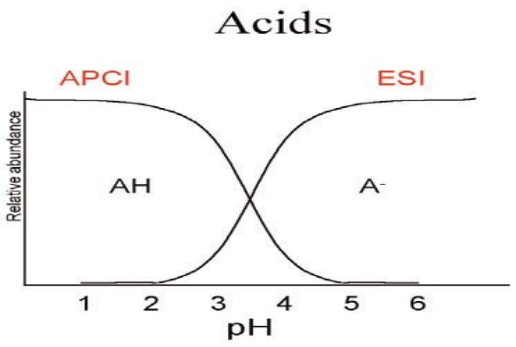
Method Development - LC/MS Solvents

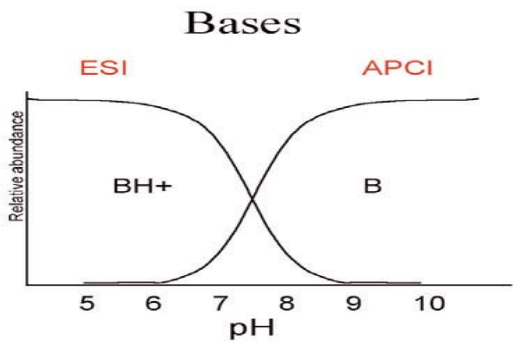
- **General Rules for selection**
  - Volatile
  - Moderate ionic strength
  - Appropriate pH

- **Solvents**
  - Water, Acetonitrile, Methanol, Ethanol, Acetone, and tetrahydorfuran (THF)
  - Buffer A- weaker more polar solvent (typically water)
  - Buffer B- stronger more non polar solvent (typically organic)

Method Development- LC/MS Additives

- **Additives**
  - Used to enhance LC Separation or Ionization
  - Common-
    - formic acid and acetic acids (0.01-1.0%)
    - ammonium formate, ammounium accetate, and ammonium hydroxide (10-20nM with ESI)
  - pH- recommend 2 units above/below pKa of molecule to enhance ionization





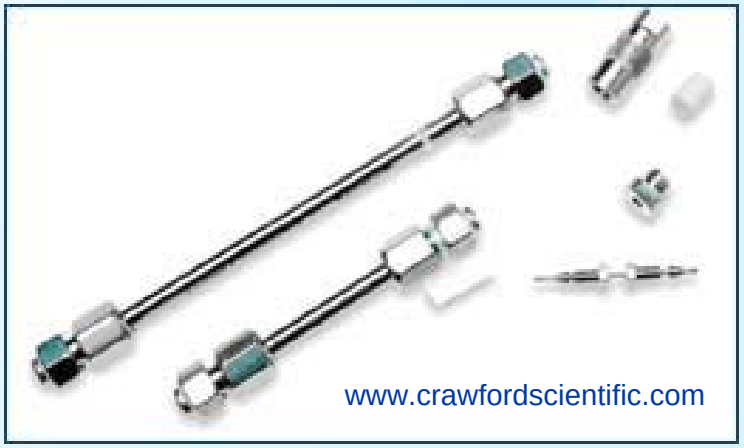
www.thermo.com/eThermo/CMA/PDFs/Various/File_29976.pdf

Method Development LC- Stationary Phase

- Common: C4, C8, C18, phenyl, phenyl-hexyl, etc.
- Particle size/column dimensions
- HPLC/UPLC (with compatible systems)
- Consider guard column to extend life of column

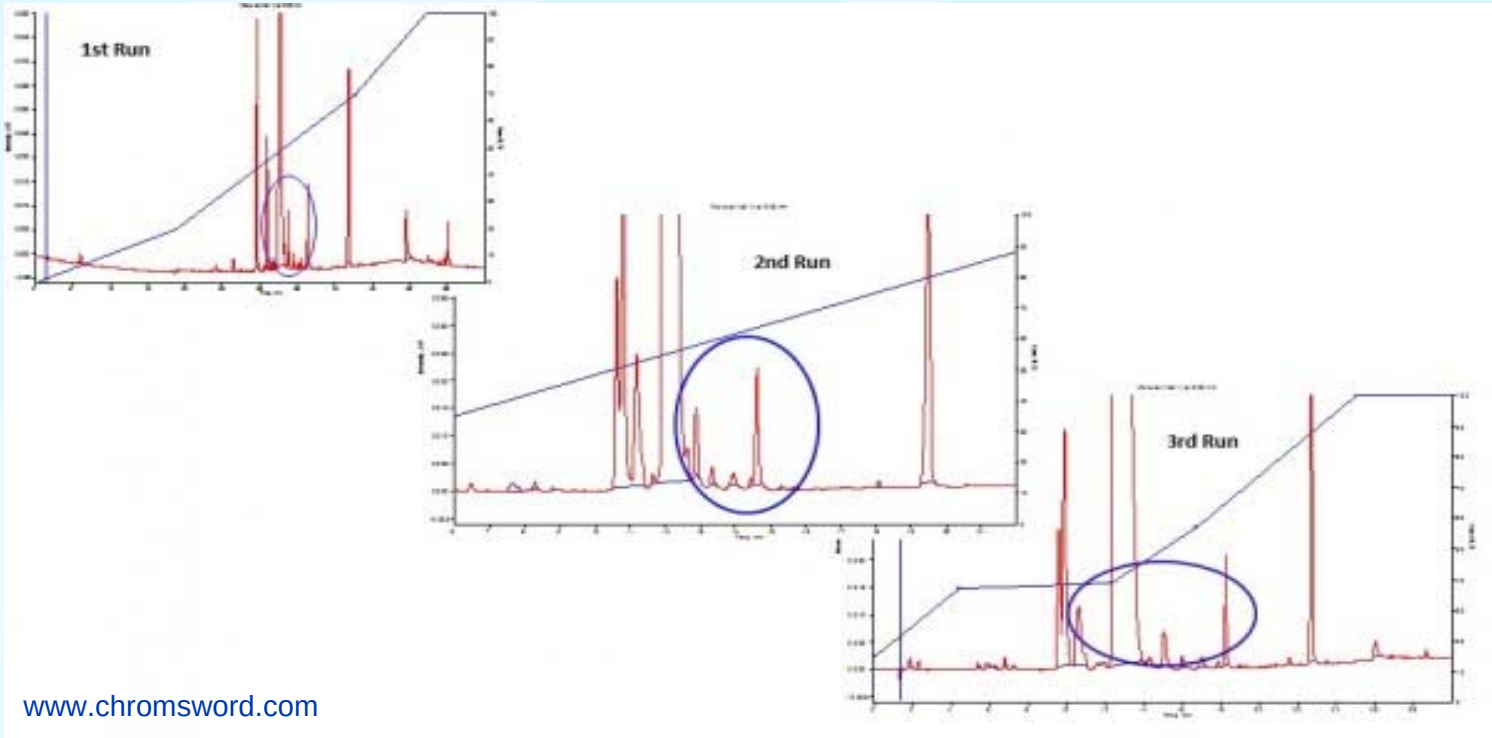
Choice depends on chemical composition of analyte

use literature and vendor support



Method Development LC Separation

- **isocratic vs. gradient elution**
  - What separates your analyte(s) from interfering compounds best?
  - Keeping in mind time/cost to get what is needed



Assay Development- LC/MS Optimization

Source Parameter Optimization-

via optimized mobile phase and flow rate with analyte

Source Gas Parameters- high as possible until significant signal loss

Temperature- higher flow rate/ higher water content = higher temperatures

Probe height

Dwell time (points across a peak 10-20)

Method Development- Preliminary Assessment LC/MS Method with Neat Compound(s)

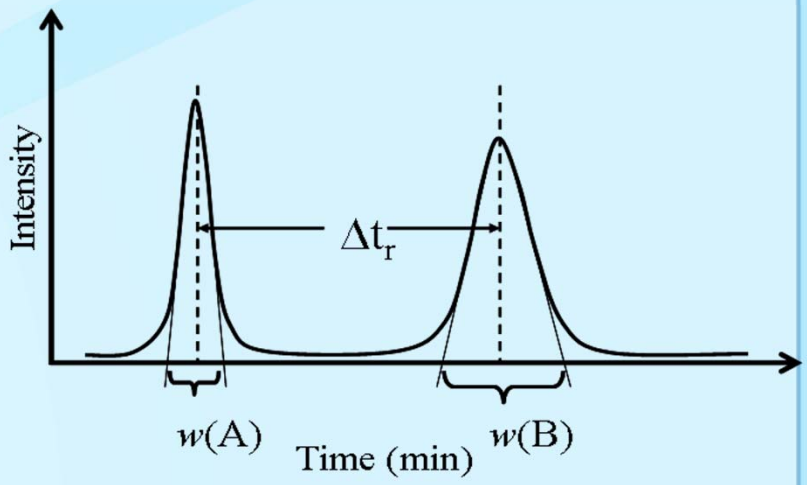
- Analyze several neat calibrators with IS *(additional points can be added)*
  - Assess imprecision at each level
  - Preliminary assessment of calibration curve (linear, reproducible)
  - Look at IS to Analyte Ratios does it look appropriate, ie IS is supposed to be 100ng/dL does it match well with 100ng/dL calibrator (ie 1:1 signal response)
  - Are QI/CI ratio of both analyte and IS stable (they are neat they should be)
  - Determine, through back calculation, approximate volume of serum needed to produce a signal with (S/N >10) in your lowest clinically relevant sample

Method Development- Sample Preparation

- Sample Volume Requirements (what is available, what you need)
- Isolation of analyte
  - Several approaches-LLE, SPE, or combination of
  - Must be compatible with LC/MS mobile phase
- More extensive clean up
  - Increases selectivity (removes interferences)
  - Increase sensitivity (removes matrix effects)
  - BUT- loss of analyte (lower extraction efficiency)
- Less extensive clean up
  - Saves time, reduces analyte loss
  - BUT- often lower precision and accuracy due to matrix effects
  - Clogged columns/Dirty instruments

Method Development- LC Optimization- Peak Resolution with samples

- Resolution of interfering compounds **with the same transition** (peaks that actually overlap)
  - Prefer baseline resolution
  - Can make Improvement with
    - Mobile phase
      - (ie change gradient)
    - different column
    - Temperature



Matrix Effect Evaluation

- **Interferences you can "see"**
  - Test know interference compounds
    - Similar mass; similar fragmentation
    - Both endogenous and exogenous compounds (i.e. bio identicals)
- **Evaluate – Test pure compounds**
  - Look for no peak or good resolution from peak at same RT as your analyte (and IS)
- **Evaluate - Use QI/CI ratios to monitor if there is a problem during development/validation and beyond**
  - In 'real' patient samples
    - including different collection tubes, disease states, ages, gender
  - Compare QI/CI to pure solution
  - Be sure to test Analyte and IS

Matrix Effect Evaluation

- **Interferences you can't "see"**
  - no peaks in the chromatogram
  - Most problematic-
    - Salts and lipids (specifically phospholipids)

- **Evaluation using Matuszewshi et al.**

  Evaluate 5 different lots of matrix- one matrix free

  Ion Suppression < 1.0 < Ion Enhancement

  MF (Matrix Factor) on analyte and IS

  - MF= Peak Area in Matrix
    Peak Area in Pure Solution (Matrix Free)

  NMF (Normalized Matrix Factor) uses Analyte/IS ratio

  - MF= Peak Area Ratio in Matrix
    Peak Area Ratio in Pure Solution (Matrix Free)

Matuszewski,et al. Analytical Chemistry. 2003;75:3019-3030

Matrix Effect Evaluation- Solutions

- **Improve sample clean up**
  - might need to sacrifice some extraction efficiency for better sample clean up
- **Improve chromatography**
  - Resolution of peaks you can ‘see’
  - Separate compounds you can’t ‘see’ to separate ion suppression compounds
- **Reconsider selected transitions**

Pre Validation

- **CLSI Document EP10A3- Preliminary Evaluation**
  - 5 day assessment
  - 3 levels (relevant to concentration range method is intended)
  - Analyzed in triplicate each day in specified order

  Outcome- assessment at 3 concentrations
  - Within, between, and overall imprecision
  - Bias, accuracy if data is available
  - Method Carryover

Pre Validation

- **If results are not acceptable, consider**
  - Imprecision to high?
    - Peak signals large enough to properly integrate?
      - optimization ionization, increase injection volume, increase sample volume, increase extraction efficiency, verify matrix effect issue
    - peak shape acceptable and reproducible
      - optimize chromatography, consider more stable transition
  - Accuracy (on limited number of samples- only 3)
    - Determine constant (all levels off by equal amount) or proportional (difference changes with concentration)
    - Investigate issue with calibrators OR selectivity (measuring an interfering compound)

Pre Validation

- **If results are acceptable**
  - Start method validation

Keeping in mind during validation and implementation if performance drops or issues come up items from method development can be very important

Acknowledgments

- **C60 Document Development Committee**
  - Dr. Bill Clark, Dr. Ross Molinaro, and Dr. Lorin Bachman
- **Dr. Sam Caudill (CDC Statistician)**

THANK YOU

Contact Information: JBotelho@cdc.gov

4770 Buford Hwy. NE, Chamblee, GA 30341
Telephone: 1-800-CDC-INFO (232-4636)/TTY: 1-888-232-6348
E-mail: cdcinfo@cdc.gov Web: www. cdc.gov