Analytical Method Validation: How & Why it is Vital?

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Clinical Research
- Conduct controlled drug administration research in human drug users
  - IRB approved, written informed consent, placebo-controlled, randomized, balanced, double blind studies
  - Multiple doses & routes of administration
  - Subjects reside on closed research unit to prevent exogenous drug administration
  - Ethical hurdles becoming prohibitive

Clinical Research
- Evaluate cognitive, subjective & physiological effects & specific brain activities (fMRI) of licit & illicit drugs
- Identify important biomarkers of drug exposure
- Fundamental hypothesis that a relationship exists between pharmacological or toxicological response to drug & concentration of drug in an accessible compartment

Clinical Pharmacokinetics
- Enables us to correlate pharmacokinetics with pharmacodynamic effects
- Enhance our understanding of physiological, biochemical, subjective & cognitive drug effects
- Controlled drug administration provides framework for understanding these processes & for interpreting drug concentrations in biological fluids
Biological fluids & tissues

- Blood
- Plasma
- Urine
- Oral Fluid
- Hair
- Sweat
- Breast milk
- Brain
- Skin

- Meconium
- Placenta
- Cord Blood
- Umbilical cord
- Amniotic fluid
- Urine
- Hair

Quantification of Drugs & Metabolites by GCMS & LCMSMS

- Irreplaceable specimens of limited volume
- High concentrations of major & low levels of minor analytes in same limited specimen
- Multiple drug classes
- Throughput & turn-around time
- Instrument time limited
- Time required to troubleshoot assay or identify interference
- Most important: producing accurate results

Method Validation

- Before you start:
  - Define validation experiments
  - Performance characteristics & acceptance criteria

Full Method Validation

- Calibration model
- Sensitivity
- Dynamic linear range
- Selectivity (Specificity)
- Bias/Recovery (Accuracy)
- Imprecision
- Extraction efficiency
- Stability
- Carryover
- Dilution integrity
Partial Method Validation
- Method transfers between laboratories
- Instrument &/or software platform changes
- Change species matrix (rat to human plasma)
- Change matrix (human urine to plasma)
- Selectivity from metabolites
- Selectivity from other medications
- Change detection systems
- Change specimen processing
- Change anti-coagulant
- Limited volume changes (pediatrics)

Cross Validation
- Comparison 2 bioanalytical methods
- One serves as reference other comparator
- Compare fortified samples & true specimens

Calibration
- Number of required calibrators
  - Generally, prefer 5 to 6 calibrators
  - Never force the curve through zero
  - Y intercept important descriptor of bias
- Linear least squares
  - Homoscedastic data (constant variance across the linear dynamic range)
- Weighted least squares (1/x)
  - Heteroscedastic data (significant differences between variances at the lowest & highest concentrations) can handle larger dynamic range

Calibration
- Quadratic (1/x2)
  - Extends the linear dynamic range
  - Requires more calibrators in the non-linear area of curve
- Historical curves
  - Not a good idea, certainly not for forensic testing
Calibration
- All methods require calculation of individual data points against the mean response factor to assess acceptability
  - Frequently ±20% of target
  - Also, ±15% of target, except at LOQ ±20% of target
- Split curves
  - Enables you to extend the dynamic range & quantify analyte concentrations with one extraction & injection
  - Required validation criteria met for both curves

Concentrations Evaluated
- Minimum of 3 quality control (QC) samples over dynamic linear range: low, medium & high
  - Low QC within 3X LOQ, middle, high end of curve
  - Some require LOQ to be tested
- Replicates of QC
  - Varies considerably
  - Minimum of 5 for intra-assay imprecision
  - Minimum of 20 for inter-assay imprecision
  - Or minimum duplicates for 10 days

Concentrations Evaluated
- Replicates of QC
  - Highly preferred to do a one-way ANOVA to estimate imprecision
- QC independently prepared from different supplier, vial or weighing than calibrators
- Volume of organic added to fortify <5%
- Number of calibration curves
  - Minimum of 4, preferable 5 or more

Sensitivity
- Limit of detection (LOD)
  - Lowest analyte concentration identified but not quantified at exact value
  - Lowest analyte concentration reliably differentiated from background noise
  - Signal to noise ratio ≥3 for analyte peak height & highest & lowest baseline noise around analyte
Sensitivity

- Limit of detection (LOD)
  - CDM requirement: empirical determination of lowest analyte concentration with acceptable chromatography, relative retention time ±2% of reference, S/N ≥3 for all ions, & ion ratios within ±20% reference
  - Reference can be from 1 or all calibrators

- Limit of quantification (LOQ)
  - Lowest analyte concentration quantified with suitable precision & accuracy
  - Signal to noise ratio of ≥10 for analyte peak height & highest & lowest baseline noise around analyte
  - CDM requirement: empirical determination of lowest analyte concentration that meets LOD & quantifies within ±20% of target
  - LOD can equal LOQ when limits determined empirically

Selectivity

- Potential endogenous matrix interference
  - Must evaluate each matrix type
  - Minimum of 6 - 8 replicates, maybe 10
  - Especially important with LCMSMS

- Potential exogenous component interference
  - Hemolysis, icteric, high lipid content
  - Metabolites
  - Concomitant medications
  - Illicit drugs

2D GCMS with Cryofocusing

Diagram of 2D GCMS with Cryofocusing showing the inlet, FID, Pneumatic Deans Switch, Cryotrap, Primary Column, and Secondary Column.
Imprecision

- Intra-assay variability (repeatability)
  - Determine CV%
  - Minimum of 5 replicates in same run
  - Preferred to perform an ANOVA to evaluate imprecision within each day of multi-day analyses
  - Preferred N=20, 5 X 4, 4 X 5, duplicates in 10 runs

- Acceptance criteria
  - ≤20%
  - If you fortify QC each day, frequently find significant differences between days
  - Report worst CV for all days & state that intra-assay imprecision significantly different, but not clinically significant

Imprecision

- Inter-assay variability (intermediate precision)
  - Differences in operator, equipment or time
  - Preferred N=20, 5 X 4, 4 X 5, duplicates in 10 runs
  - Acceptance criteria ≤20%

- Reproducibility
  - Precision between laboratories (external proficiency test results)
  - Standardizing between laboratories

Carryover

- Contamination by a preceding sample
- During validation, establish concentration above which contamination could occur
- Test highest contamination that is expected & work lower as needed to establish this limit
- Inject each concentration followed by zero specimen (can quantify interference)
- If follow by blank or solvent, only eyeball results
- Typically expect result <LOD
**Carryover**

- If carryover concentration is above the linear range of method, can’t be sure of concentration
- Typically, determine no carryover at upper LOQ
- Should re-extract following specimen due to potential carryover into the vial
- Some suggest reinjection & compare 2 quantifications
- Could also reinject high specimen followed by zero specimen to quantify amount of carryover

**Recovery**

- Accuracy is difference between test result & true result
- Requires comparison against certified reference standard
- In many toxicological methods, reference standard not available (exception blood alcohol, some TDM)
- Generally, method bias or analyte recovery determined
- Measure how mean value of experimental observations agrees with “accepted value”
- Little agreement on appropriate method for recovery

**Recovery**

- Two solution approach
  - Prepare 10 tubes with identical blank matrix
  - Fortify 5 tubes with native analyte ($d_0$) & deuterated internal standard ($d_3$) prior to extraction
  - Extract 5 tubes & fortify with $d_0$ & $d_3$ after extraction
  - $\%$ recovery $d_0 = \frac{\text{mean target ion area pre-extraction}}{\text{mean target ion area post-extraction}}$
  - $\%$ recovery $d_3 = \frac{\text{mean target ion area pre-extraction}}{\text{mean target ion area post-extraction}}$

- Acceptance criteria: as close as possible recovery for $d_0$ & $d_3$

- Advantages
  - Accounts for variability between SPE tubes, injection volume & other factors
  - Useful for non-deuterated internal standards
  - Only requires 2 tubes
Recovery

Three solution approach
- Prepare 6 tubes with identical blank matrix
  - A: fortify 3 tubes with d₀ & d₃ prior to extraction
  - B: fortify 3 tubes with d₀ prior to extraction; add d₃ after extraction
  - C: Prepare a neat solution of same amount of d₀ & d₃
- Extraction = mean ratio d₀ & d₃ areas pre-extraction efficiency / mean ratio d₀ & d₃ areas post-extraction
- Absolute = mean target ion area pre-extraction recovery / mean target ion area post-extraction

Accuracy vs Precision

Not accurate Not precise Accurate Precise

Stability

Analyte stability in a given matrix under specific conditions for given time intervals
- During storage prior to analysis
  - Room temperature, 4°C, -20°C, -80°C
- Of derivatized extracts
- On autosampler
- 3 freeze-thaw cycles
- All conditions should be determined for realistic storage times

CDM stability requirements

3 QC levels (3 replicates) quantify ±20% compared to freshly prepared & analyzed specimens
- Room temperature for 8 h
- 1 - 7 days at 4°C
- 1 to 12 wks -20°C (rarely -80°C)
- Derivatized extracts for 48 to 96 h (temperature?)
- On autosampler for 48 to 96 h
- 3 freeze-thaw cycles
- Our operation not designed for long-term stability studies- limitation
Dilution integrity

- Document dilution protocol produces accurate results
- Dilute high concentration QC with blank matrix to bring concentration within analytical range
- Document that results ±20% of target
- Good practice during routine analysis to always dilute a QC with patient specimen
- If water or buffer are used to dilute, must validate that results are equivalent

Robustness

- Measures method susceptibility to small changes occurring during routine analysis
  - pH
  - Mobile phase composition
  - Flow rate
  - Column temperature
  - Lots of extraction materials, columns, solvents
  - Derivatization temperature
  - Different analytes, instruments

LCMS, LCMSMS & LCMS-TOF

- Ion suppression (↓ analyte signal) & ion enhancement (↑ analyte signal) due to matrix effects
- Matrix effect due to alteration of ionization efficiency due to presence of co-eluting ions
- Exact mechanism unknown
- Competition between non-volatile matrix ions & analyte ions for transfer to gas phase
- Greater problem with electrospray ionization than atmospheric pressure chemical ionization

LCMS, LCMSMS & LCMS-TOF

- More suppression with polar ions elute early with many matrix components
- Post-extraction addition of analytes
  - Compare analyte areas in matrix & mobile phase
  - Static method: data about matrix effects at time of analyte elution
- Post-column infusion:
  - Infuse analyte continuously into MS, inject matrix after different specimen preparation techniques
  - See areas of matrix effect across run time
**LCMS, LCMSMS & LCMS-TOF**

- **Additives in mobile phase**
  - Acetate vs trifluoroacetate (reduces ionization)
  - Phospholipids produce ion suppression
    - Glycerophosphocholines major type in plasma
    - Monitor 184 to 184 ion to see phospholipids
  - In routine practice can help determine when necessary to change analytical column
- **Specimen containers**
- **Preservatives**

**LCMS, LCMSMS & LCMS-TOF**

- **Minimize matrix effect**
  - Improve specimen preparation
  - Change chromatography to move analytes of interest to area of chromatogram without effects
  - Choose different ion source
  - Deuterated internal standard minimizes matrix effect
    - Need internal standard that mimics extraction efficiency from matrix & ionization efficiency

**LCMS, LCMSMS & LCMS-TOF**

- **Matuszewski et al Anal Chem 2003;75:3019-30**
  - Suggested assay calibration curve in at least 6 blank matrices & compare %CVs
- **Taylor LCMS Method Validation IATDMCT 07**
  - Suggested assay 5 replicates of 3 QCs in 5 different specimen matrices
  - Add $d_0$ & $d_3$ pre & post-extraction & in mobile phase
  - 45 injections for matrix effect, absolute recovery, process efficiency & inter-subject variability

**LCMS, LCMSMS & LCMS-TOF**

- **Taylor IATDMCT 2007**
  - Matrix effects: Post - Pure/Pure *100
  - Absolute recovery = Pre/Post *100
  - Process efficiency: Pre/Pure *100
  - Inter-subject variability: CV of 5 Pre samples
- **Huestis**:
  - Absolute recovery = Pre/Pure *100
  - Process efficiency: Pre/Post *100
  - CDM uses ratios rather than area abundance to remove variability by including internal standard
Useful References

- Guidance for Industry: Bioanalytical Method Validation
  [http://www.fda.gov/cder/guidance/4252fnl.htm](http://www.fda.gov/cder/guidance/4252fnl.htm)
- Clinical & Laboratory Standards Institute (formerly NCCLS)
  [http://www.nccls.org/](http://www.nccls.org/)
- International Conference on Harmonization: Chemical & Pharmaceutical Quality Assurance
- International Organization for Standardization
  - ISO 9000 Quality Management Principles
  [http://www.iso.org/iso/home.htm](http://www.iso.org/iso/home.htm)
- Shah et al 2000 *Pharmaceutical Research* 17:1551-1557
- Matuszewski et al *J Chrom B* 2006;830:293-300

Analytical Method

- Develop & validate a sensitive & specific analytical method for detection & quantification of MDMA & metabolites MDA, HMMA & HMA
- 2D-GC/MS for sufficient sensitivity & specificity
- LOQ ≤ 10 ng/mL necessary for characterizing pharmacokinetics of MDMA & metabolites
- To analyze specimens from controlled MDMA administration study
- Erin Kolbrich’s doctoral dissertation

MDMA GCMS Analytical Method

- Acid hydrolysis - 0.5 M HCl (time, temp & amt)
- pH adjust - 0.1 M phosphate buffer & 10 M NaOH
- SPE- UCT Styre Screen™ DBX
- Derivatization- 10 µL HFBA
- Back extract - 200 µL TRIS buffer
- 3 µL splitless GC injection
Analytical Method

- 2D-GC/MS
  - Instrument: Agilent 6890N GC with 5973 MSD
  - GC:
    - 1° Column: Agilent DB1-MS (non-polar)
    - 2° Column: Phenomenex ZB-50 (↑ polarity)
  - Three “cut” windows
  - Cryotrap: 50°C, then 800°C/min to 275°C
  - Oven: 70-195°C, 195-100°C (fast), 100-190°C
- MS
  - Ionization: electron impact
  - Mass transfer line temperature: 280°C

Analytical Method Validation

- 3 quality control concentrations/analyte
  - 2.5, 15, 75 ng/mL MDA & HMA
  - 7.5, 40, 200 ng/mL MDMA & HMMA
- LOD & LOQ
- Bias/Analytical recovery “Accuracy”
- Imprecision
- Extraction efficiency
- Specificity
- Dilution integrity, stability & carryover

Analytical Method Validation

- Sensitivity & Linear dynamic range

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Internal standard</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
<th>Linear range (ng/mL)</th>
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<td>HMA</td>
<td>Pholedrine</td>
<td>0.5</td>
<td>2.5</td>
<td>2.5 - 100</td>
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</table>

- 6 concentrations per curve, except HMA = 5
- Pholedrine as IS
- $R^2 \geq 0.997$
### Analytical Method Validation

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Target (ng/mL)</th>
<th>Intra-assay mean % CV (n = 5 replicates/run, total of 4 runs)</th>
<th>Inter-assay % CV (n = 20)</th>
<th>% of Target (n = 20)</th>
<th>% (n = 5)</th>
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### Analytical Method

#### Dilution integrity
- 75% dilution with buffer
- All within ± 20% of ¼ mean high QC

#### Stability
- Short-term: 12 h room temp, 48 h refrigeration
- Long-term: 3 freeze-thaw cycles
- All within ± 20% of fresh QC, except low HMA

#### Carryover
- Low QC injected after 1000 ng/mL
- Quantification & ion ratios within ± 20% target

### Analytical Method

#### Interfering compounds
- Cocaine
- Benzoylcegonine
- Norcocaine
- Norbenzoylcegonine
- Egonine ethyl ester
- Egonine methyl ester
- Anhydroecgonine methyl ester
- Egonine
- 6-Acetylmorphine
- Morphine
- Normorphine
- Morphone-3-glucuronide
- Morphone-6-glucuronide
- Codeine
- Norcodeine
- 6-Acetylcodine
- Hydrocodone
- Hydromorphone
- Oxycodone
- Clonidine
- Pentazocine
- Caffeine
- Imipramine
- Clopramide
- Fluoxetine
- Norfluroxetine
- Paroxetine
- 7-Aminoclonazepam
- Clonazepam
- Dihexyamine
- Phenylpropanolamine
- Pseudoephedrine
- Norpseudoephedrine
- Diazepam
- Lorazepam
- Oxazepam
- Alprazolam
- Flunitrazepam
- Temazepam
- THC
- Buprofen
- Acetylsalicylic acid
- Acetaminophen
- Nicotine
- Gammahydroxybutyrate
- Phentermine
- Fenfluramine
- Dextromethorphan
- p-Hydroxyamphetamine
- p-Methoxyamphetamine
- 11-OH-THC

### Analytical Method

#### Participant specimen: 7 h after 106 mg MDMA dose

- MDMA: 4.1 ng/mL
- HMA: 110.3 ng/mL
- MDEA: 80.9 ng/mL
Advantages of LC/MS/MS

- Simple specimen preparation
  - Some matrices are dilute & shoot (urine)
  - Protein precipitation with ACN (blood, oral fluid)
  - No derivatization
- Wide range of analytes in a single assay
- Thermolabile & nonvolatile analytes
- Potential for eliminating hydrolysis step & directly analyzing phase 2 metabolites
- Large dynamic linear range
- Improved fragmentation of opioids

Disadvantages

- Cost
- Knowledge & expertise
  - Less than for GC/MS
  - More than for GC/MS/MS
- Matrix effect
  - Suppression & enhancement
  - Necessary to quantify effect
  - Lack of deuterated internal standards for minor metabolites & glucuronides problematic

Opiates, Cocaine, & Metabolites in Oral Fluid

- Why LC/MS?
  - Simple specimen preparation
    - Protein precipitation with acetonitrile
    - No derivatization
  - 26 cocaine & opiate analytes in a single run
  - Atmospheric pressure chemical ionization
  - 200 µL oral fluid specimen
  - Matrix matched calibrators 1 - 500 µg/L
  - Intra-assay imprecision <15.8%; inter-assay imprecision <22.8%

- Demonstrates typical concentrations in oral fluid after illicit use
- Applicability of SAMHSA cutoff concentrations for detecting exposure
- Evaluates effectiveness of drug treatment
- Determined drug recovery from oral fluid collection device & oral fluid volume recovery
- Monitored wider range of analytes
Thank You for Your Attention!