Challenges and Solutions For Regulated Bioanalysis Using SPE-UPLC/MS/MS

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Agenda

- Improvements in the Method Development Process
- Quantification of Low Exposure Drugs
- Regulatory changes and their impact on bioanalysis methodology
  - Matrix effects
  - ISR
  - Metabolites in Safety Testing
Requirements For A Successful Bioanalytical Assay

- Selectively detect and quantify analyte using a linear response detector
- Isolate analytes from matrix
- Resolve analyte from metabolites
Challenges in Developing A Bioanalytical Assay

Sample Prep
- Removal of proteins and other endogenous material
- PPT, LLE, SPE

LC
- High throughput separations method
- Isocratic, reversed-phase, gradient ??

MS/MS
- MS MRM method development
- Mode of ionization, best precursor and product ions
Sample Preparation Options

- PPT
- LLE
- SPE
- PLR Plate

Highest Selectivity
- Direct Inject
- Simple

High throughput
- PL Removal

Highest Sensitivity

“Clean” Extracts
Options in Sample Preparation

SIMPLY CLEAR

THE FASTER WAY TO CLEANER

SENSITIVITY IN ITS PUREST FORM

Sivacca™ Protein Precipitation Plate

Ostro™ 96-Well Plate

Oasis™ RLE/Electron Plate

Sample Extraction Products

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Comparison of Techniques: Lipid Levels

MRM 184 -> 184

LLE w/ 5% NH₄OH

LLE

PLR plate

RP SPE

PPT
Oasis SPE Reduces Matrix Effects Consistently

Protein precipitation
Background ms scan

Protein precipitation
Monitor Alprazolam
MRM 309->281

RAZ_SPE_058

SPE Background ms scan

ALPRAZ_SPE_058

1: MRM 309.2

SPE Monitor Alprazolam
MRM 309->281
μElution Plate Benefits

μElution plate

Elution volume 2x25ul, diluted 50ul H2O

Sensitivity: 5pg/ml

30mg Oasis plate

1: MRM of 2 Channels ES+
309.2 > 281 (Alprazolam)
8.10e6 Area

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309.2 > 281 (Alprazolam)
8.10e6 Area
Resolution – Comparison of HPLC and UPLC Selectivity

Background

Analyte MRM

Greater Sensitivity from UPLC system due to resolution from endogenous matrix
BEH UPLC Technology
Using pH to Increase Resolution

Ranitidine   Plasma

Basic Separation

Greater peak retention

Acidic Separation

Ranitidine   pKa 8.2, 2.7

H₃C
\[\text{N} \equiv \text{H} \]

H₃C
\[\text{N} \equiv \text{H} \]

\[\text{S} \quad \text{\text{H}₃C} \quad \text{N} \quad \text{\text{CH₃}}\]

1: MRM of 1 Channel ES+ TIC (Ranitidine) 2.24e5
2: MS2 ES+ TIC 1.84e9
3: Parents of 184ES+ TIC 1.35e9

17MAR2010_PR_119

17MAR2010_PR_103
Method development: Optimization of MS Conditions

**Infusion Set-Up**

- Line from LC pump, contains mobile phase
- Line from infusion pump, contains analyte
- Probe may need to be changed

**Xevo Set-Up**
MRM Method Development

**IntelliStart Process**

- Input the compound name
- Calculate potential precursor masses
- Input the monoisotopic precursor mass(es)
- Limit low mass fragments
- Click Start
- Produces report and MRM method
Xevo TQ-S

Ultra high sensitivity for targeted quantification
Xevo TQ-S Fluticasone (0.75pg/mL in Plasma, or 1.7pM)
How did we increase sensitivity?

Xevo TQ-S

Larger sampling orifice

Problems associated with a larger sampling orifice:

1) A disperse ion cloud
2) Higher levels of matrix contamination
Designed to deal with problems associated with a larger sampling orifice

Maximising signal

Maximising robustness
Blank Extracted Chromatogram & Reproducibility Data

Blank

0.75pg/mL Standard

<table>
<thead>
<tr>
<th>Analte Concentration pg/mL</th>
<th>Standard Deviation</th>
<th>Mean</th>
<th>% CV</th>
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<tbody>
<tr>
<td>0.75</td>
<td>0.04</td>
<td>0.67</td>
<td>6.05</td>
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<tr>
<td>1.00</td>
<td>0.07</td>
<td>0.98</td>
<td>7.33</td>
</tr>
<tr>
<td>1.50</td>
<td>0.04</td>
<td>1.31</td>
<td>2.70</td>
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<tr>
<td>3.75</td>
<td>0.19</td>
<td>3.67</td>
<td>5.06</td>
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<tr>
<td>5.00</td>
<td>0.15</td>
<td>5.29</td>
<td>2.75</td>
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<tr>
<td>7.50</td>
<td>0.24</td>
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<td>3.16</td>
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<td>10.19</td>
<td>4.13</td>
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<td>15.00</td>
<td>0.37</td>
<td>14.75</td>
<td>2.51</td>
</tr>
</tbody>
</table>
Xevo TQ-S Salmeterol Succinate (0.5pg/mL in Plasma, or 1.2pM)

M RM of 4 Channels ES+
416.3 > 232.1 (salmeterol)
2.94e5

Compound name: salmeterol
Correlation coefficient: $r = 0.999728$, $r^2 = 0.999455$
Calibration curve: $1.13292 \times x + 4.11881$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axes trans: None

Salmeterol Succinate (0.5pg/mL in Plasma, or 1.2pM)
In incurred sample reanalysis, method consistency is crucial. Study samples need to be reanalyzed to ensure method reliability.

- Matrix composition varies significantly between species and patients, influenced by diet, age, gender, and disease state.
- Resolution from the matrix is critical.
- Matrix effects must be calculated and quantified.
- MIST guidelines from the FDA require any human drug metabolite formed “at greater than 10% of parent drug systemic exposure at steady state” to undergo separate safety testing.
Gradient method 1: 5-95% MeOH in 0.70 minutes

Gradient method 2: 5-95% MeOH in 2.0 minutes

Fluticasone 501>293

Radar Solvent Blank

Radar Plasma Blank

Phospholipids 184>184
Automating Matrix Factor Calculations: Integrated Intellistart Fluidics
MassLynx TargetLynx Simplifies Calculating The Matrix Effects

TargetLynx™ automatically calculates matrix effects from 6 injections

Matrix Calculation Without Convolution

Matrix Calculation With Convolution

Results Presented in Final Report
Simultaneous MRM and Full Scan Detection

MRM of Parent

Full Scan of Metabolites
Alternative approach to full-scan profiling of metabolites

- Screen for selected panel of metabolites
  - Commonly reported metabolites
  - User definable
- Predicts metabolite MRMs based upon Parent Compound MS/MS, obtained from PICS in MRM raw data file
- Generates MANY MRMs
  - 1 msec dwell time
- With RADAR, full scan MS may be added to the experiment
Develop Theoretical MRMs to Investigate

- MSMS of Parent Compound
- Identify Product Ions From Parent Compound
- Select Metabolites from list of probable transformations
- Calculate MRMs and Write MS Method File
- List of Potential Metabolites from Text File
- iMRM Method
- Automatic Product Ion Selection
- Spectra of Parent Automatically Obtained from MassLynx
The image shows a screenshot of a software interface for metabolomics method development. The interface includes a table and a graph. The table lists several metabolites with their respective retention times and ionization modes. The graph appears to display chromatograms for these metabolites over time.

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**Table:**

<table>
<thead>
<tr>
<th>No.</th>
<th>Type</th>
<th>Information</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>MRM 442.20</td>
<td>PIC, Time 0.00 to 12.00, ES+ (Dosed_Compound_442_Parent)</td>
</tr>
<tr>
<td>2</td>
<td>MRM 442.20</td>
<td>PIC, Time 0.00 to 12.00, ES+ (Dosed_Compound_442_Parent)</td>
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<tr>
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**Graph:**

The graph shows the chromatograms of the metabolites over time, with the x-axis representing time and the y-axis representing peak intensity. The colors indicate different retention times and ionization modes.
Comparison of MRM to Full Scan Analysis

Full Scan Mode Data

<table>
<thead>
<tr>
<th>Group 6 32 3 hr</th>
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<tbody>
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<td>17_4_005a</td>
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</table>

XIC 416.15

Dealkylation

MRM 416.15>325.85

XIC 458.20

Hydroxylation

MRM 458.20>339.85

XIC 428.18

Demethylation

MRM 428.18>325.83

XIC 442.20

Parent

MRM 442.2>325.85

MRM Mode Data
Simple Metabolite Detection and Confirmation

- [Benefits]
  - Metabolites detected with maximum sensitivity
  - Spectra acquired in the time scale of UPLC peak
  - Data acquired in one analytical run, no need for confirmatory experiment
Conclusion

Combination of Waters Sample Preparation and LC/MS technologies provide class leading performance

Xevo TQ-S Stepwave technology give maximum assay Sensitivity

UPLC resolution and ScanWave capability allows detection and identification of metabolites

Complete System Solution with SPE, UPLC and Xevo TQ-S facilitates the reduction and automatic measurement of matric effects