Sample Preparation for Bioanalysis

Barcelona, 7 de Junio de 2012
Outline

- Introduction
  - Challenges in Bioanalytical Method Development
- Sample preparation techniques
  - Waters’ solutions
- Application examples
Segments Within Bioanalysis

**Discovery**
*(Candidate Selection)*
- Thousands of cmpds
- Average sensitivity
- Clear/Clean extract
- Easy to use
- Reliable
- Non-regulated

**Development**
*(Pre-clinical)*
- Several cmpds
- Better sensitivity
- Clean/Pure extract
- Method development
- Reliable and inter/intra reproduc.
- Regulated

**Late Phase**
*(Phase 1-3 Clinical)*
- Targeted cmpds
- Highest sensitivity
- Pure extract
- Reliable and inter/intra reproducibility
- Regulated

- Sometimes “methods” linked
- “Methods” always linked

Leads to different testing patterns
Our Approach to Bioanalytical Method Development

- No “one size fits all”
- Different segments of drug development process
  - Scientific and business drivers may be different
  - Drivers may be the same but with varying degrees of risk tolerance
- Use of scientifically appropriate criteria for final method choice
Goals of Sample Preparation

Minimize risk
- Minimize matrix effects
  - Reduction of ion suppression/enhancement, interferences, background
- Eliminate sample to sample variability
  - More reproducible quantitation
  - More robust assays
  - i.e., Plasma from different subjects or species
- Decrease assay variability
  - Pass ISR
  - Successful transfer across labs, analysts, sites

Increased sensitivity
- Sample concentration
- Removal of interferences

Cleaner Samples
- Increased instrument uptime
- Improved method robustness
## Different Methods for Different Purposes: Decision Making Process

<table>
<thead>
<tr>
<th></th>
<th>PPT</th>
<th>PLR Plate</th>
<th>LLE</th>
<th>SPE</th>
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<tbody>
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<td>“Clean” Extracts</td>
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1. The simplest method which meets the assay needs is usually chosen
   - PPT or LLE are common starting points

2. For challenging assays (low detection limits, closely related endogenous constituents, inhalation products, peptides, etc) SPE may be first choice

3. Exact technique chosen will depend on outcome of study and how much risk can be tolerated
Sample Prep products for bioanalysis

SIMPLY CLEAR
THE FASTER WAY TO CLEANER
SENSITIVITY IN ITS PUREST FORM
Ostro is a sample preparation device for Bioanalytical scientists who want to remove phospholipids from their sample. Unlike alternative techniques such as Liquid Liquid Extraction (LLE) and Protein Precipitation (PPT), Ostro uses a combination of filtration and sorbent interaction to give scientists a cleaner sample in less time.
What is Ostro?

- 96-well plate for phospholipid removal
  - Plasma, serum samples (samples tested thus far)
    - Bioanalysis, Clinical
- Utilizes in-well protein crash
  - Sorbent interaction and filtration
  - Silica-based sorbent with C18 bonding in proprietary coverage that retains phospholipids

1. Add plasma
2. Add crash solvent
3. Mix

Proprietary frits – allow in-well PPT

- 25 mg proprietary sorbent – Removes phospholipids, analytes pass through

4. Collect Sample
5. Inject into LC/MS
How does Ostro Work?

- Methodology

1. Place Ostro onto collection plate
2. Pipette 50-200µL of plasma into wells
3. Forcefully add 1% formic acid in acetonitrile, 3:1 solvent:plasma (methanol not recommended)
4. Mix thoroughly by aspirating 3x with pipette
5. Filter samples using vacuum manifold or positive pressure manifold
6. Analyze samples

It is possible to work with lower sample volumes (such as 25µL). When doing so you will need a higher organic solvent to sample ratio, such as 10:1 or 20:1.

The well volume is 1.9 mL, however in order to mix by aspiration, the maximum volume is 1.4 mL. This translates to a maximum sample size of 350µL.
Hydrophilic-Lipophilic Balanced Copolymer

- Water wettable
- Polar retention
- Stable across pH 1-14
- No silanol interactions
- High recoveries for acids, bases and neutrals
Oasis Solid-Phase Extraction (SPE)

- **Oasis® MCX**
  - pKa < 1
  - 1 meq/g
  - Hydrophilic Retention of Polars
  - Lipophilic RP Retention

- **Oasis® HLB**
  - Hydrophilic Retention of Polars
  - Lipophilic RP Retention

- **Oasis® MAX**
  - pKa > 18
  - 0.25 meq/g

- **Oasis® WAX**
  - Water-wettable
  - Stable across pH 0-14
  - No silanol interactions
  - pKa ~ 6
  - 0.6 meq/g

- **Oasis® WCX**
  - pKa ~ 5
  - 0.75 meq/g
Comparison of Formats

**Cartridges**
- Typical load: 0.5-1000 mL of undiluted sample
- Minimum elution volume: typically 0.5 mL
- Evaporation and reconstitution necessary for concentration

**Traditional Plate**
- Typical load: 250-1000 μL of undiluted sample
- Minimum elution volume: typically 200 μL
- Evaporation and reconstitution necessary for concentration

**μElution Plate**
- Typical load: 25-375 μL of undiluted sample (≈50-750 μL diluted sample)
- Minimum elution volume: 25 μL
- No evaporation and reconstitution necessary
Novel plate technology enables **25 µL** SPE elution
- Not possible in disk/membrane products
- Allows loading sample volumes from **25 to a maximum of 375 µL**
  - 50 to 750 µL 1:1 diluted sample, 750 µL is the well volume
- Elution volume in as little as 25 µL

No evaporation means higher **throughput** and **sensitivity**
- Sensitive and selective SPE for bioanalytical clinical samples
- Increased sensitivity: up to 15x concentration factor (through format change)

**SPE without an evaporation step**
Why Oasis® µElution Format?
Sample Enrichment:
Up to a 15X Concentration Factor

0.5 ng/mL risperidone

MCX µElution plate

15X concentration

MCX 10 mg plate

No concentration
**SPE Methodology Oasis® HLB:**

**Protocol**

**Prepare Sample**
- Prepare Sample

**Condition/Equilibrate**
- Load Sample

**Wash:**
- Wash:
  - 5 % MetOH in Water

**Elute:**
- Elute:
  - ACN:MeOH 60:40

**Dilution with H3PO4 (final conc 2 %)**
- 200 ul MetOH/200 ul Water
- xx ul sample
- 200 ul
- 2 x 25 ul
Simple Straightforward SPE Methodology

Oasis® 2x4 Method:

For Bases:
pKa 2-10
Use Oasis® MCX

For Strong Acids:
pKa <1.0
Use Oasis® WAX

For Strong Bases:
pKa >10
Use Oasis® WCX

For Acids:
pKa 2-8
Use Oasis® MAX

Protocol 1

1. Prepare Sample
2. Condition/Equilibrate
3. Load Sample
4. Wash: 2% Formic acid (A)
5. Elute 1: 100% MeOH
6. Elute 2: 5% NH₄OH in 60:40 ACN:MeOH (D)

Protocol 2

1. Prepare Sample
2. Condition/Equilibrate
3. Load Sample
4. Wash: 5% NH₄OH (C)
5. Elute 1: 100% MeOH
6. Elute 2: 2% HCOOH in 60:40 ACN:MeOH (B)

Evaluation Group; CO R & D

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Example 1: Amitriptyline and Imipramine

**Imipramine**
MW 280.4  
pKa = 9.5

**IS: Amitriptyline**
MW 277.4  
pKa = 9.4

**Assay Use**
Routine sample analysis, patient screening

**Assay Requirements**
- Very high throughput
- LLOQ 50pg/mL
- Lab is concerned about system robustness and build up of phospholipids on LC columns and in MS source
- Direct injection to speed up workflow
- Simplest sample prep possible
Recoveries > 85%, M.E. < 30%

Ostro: Results obtained

Compound name: Imipramine
Correlation coefficient: r = 0.999854, r^2 = 0.999707
Calibration curve: 0.337053 * x + 0.00290389
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axes trans: None

<table>
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<tr>
<th>Standard conc. ng/mL</th>
<th>Imipramine Area</th>
<th>IS Area</th>
<th>Response</th>
<th>Calc. conc. ng/mL</th>
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</table>
Choice of Technique: Lipid Levels

MRM 184 -> 184

LLE w/ 5% NH₄OH

LLE

PLR plate

RP SPE

PPT
Lipid Level Comparison Between PPT and PL Removal Plate

PPT

PL Removal Plate

184 -> 184

184.4 > 184.4 (Lipid 184)

5.63e7

1.69

1.64

PL Removal Plate

496.4 > 184.4 (Lipid 496)

1.34e8

2.02

2.02

496 -> 184
Example 2: Oxycodone and D6 IS in Plasma

Oxycodone
MW 315.4
pKα = 8.5

IS:
D-6 Oxycodone
MW 321.4
pKα = 8.5

Assay Use
Routine analysis or screening of patient samples, GLP or clinical lab

Assay Requirements
- LLOQ 50 pg/mL
- Simple method
- Method must work for urine too
- Must transfer across lab with varying levels of expertise
- Metabolites and related compounds need to be cleaned up and quantitated also
Recoveries > 85% and M.E. < 25% (2 matrices, 2 analytes)

<table>
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<tr>
<th>Standard conc. ng/mL</th>
<th>Oxycodone Area</th>
<th>IS Area</th>
<th>Response ng/mL</th>
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</table>

50 pg/mL oxycodone in extracted plasma

Blank plasma

All standards and QC samples easily meet regulatory criteria
Example 3: Ropinirole and IS

**Assay Use**
Regulated analysis of patient samples

**Assay Requirements**
- LLOQ 5 pg/mL
- Highest selectivity method possible
- Free from closely related endogenous interferences
- Prefer to concentrate without evaporation
Recoveries higher than 90% and M.E. lower than 5%

Basic Mixed-mode SPE Extraction Method
Easily Meets LLOQ of 5 pg/mL
Assay Use
Regulated analysis of patient samples

Assay Requirements
- LLOQ 1-5 pg/mL
- Highest selectivity method possible
- Free from closely related endogenous interferences
- Challenging detection limits
- Need to concentrate without evaporation
Path to Peptide SPE Screening Protocol

**Original Protocol**

- **Oasis® WCX μElution**

  - Dilute plasma with 4% H₃PO₄
  - Condition MeOH/Equilibratate H₂O
  - Load Diluted Plasma

  - **Wash 1:** 5% NH₄OH
  - **Wash 2:** 100% MeOH
  - **Elution:** 2% FA in 60/40 ACN/MeOH
  - **Dilute:** 0.1% TFA

**Optimized Protocol**

- **Oasis® MAX μElution**

  - Dilute plasma with 4% H₃PO₄
  - Condition MeOH/Equilibratate H₂O
  - Load Diluted Plasma

  - **Wash 1:** 5% NH₄OH
  - **Wash 2:** 20% ACN
  - **Elution:** 1% TFA in 75/25 ACN/H₂O
  - **Dilute:** H₂O
Dilute plasma with 4% $\text{H}_3\text{PO}_4$

Condition MeOH/Equilibrate $\text{H}_2\text{O}$

Load Diluted Plasma

- **Wash 1:** 5% $\text{NH}_4\text{OH}$
- **Wash 2:** 20% ACN
- **Elution:** 1% TFA in 75/25 ACN/$\text{H}_2\text{O}$

Dilute: $\text{H}_2\text{O}$
SPE Recoveries Using Peptide Screening Protocol

Great results for diverse peptides: Screening protocol results in method for 75% of peptides!
Final SPE Results after BNP, Enfuvirtide and Somatostatin Methods Optimized

Minor, compound specific, modifications for 3 peptides result in excellent recovery for all peptides
Desmopressin: Oasis WCX

Recovery higher than 90% and M.E. lower than 5%

Calibration curve: 1.5469 * x + 0.000270265

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Std. Conc</th>
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<th>IS Area</th>
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350 µL human plasma

MRM of 1 Channel ES+
433.1 > 513.2 (Angiotensin I)

1.54e3
Area

Time

0.25 0.50 0.75 1.00 1.25 1.50 1.75 2.00 2.25 2.50 2.75

0.86
26

10 pg/mL

0.85
19

5 pg/mL

0.87
12

1 pg/mL

0.86
3

Blank plasma

©2011 Waters Corporation
350 µL human plasma

- 5 pg/mL
- 1 pg/mL
- Blank plasma

MRM of 2 Channels ES+
349.8 > 263 (Angiotensin II)
3.64e3 Area

Evaluation Group; CO R & D
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Bioanalysis of peptides

- In-house seminars
- Peptides’ video:
  
  www.waters.com/pepDVD
- Peptides’ day
- ... and more
Conclusions

- Methods are not one-size fits all

- Depending on the complexity of the particular assay Waters offers different solutions: PPT, PLR and SPE

- Mixed-mode SPE facilitates routine achievement of low pg/mL LLOQ’s for both large and small molecules
Questions?
Recent Regulatory Discussions: Incurred Sample Reanalysis (ISR)

- Incurred Sample Reanalysis (ISR) discussions at recent Crystal City Meeting, February, 2008; followed by CVG (Canada), EBF (Europe)
  - ISR will be required

  - Must have SOP for ISR in place

  - A % of study samples will need to be reanalyzed

  - Various acceptance criteria were discussed and a consensus proposed

  - AAPS Journal paper published describing one such approach to ISR and determination of criteria\(^3\)
    - One particular proposal for acceptance criteria that was suggested by various AAPS representatives is the use of the 4/6/20 rule\(^4\)
    - Two thirds of repeats must be within 20% of the original value

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\(^1\)AAPS Journal 2007: 9 (1) Article 4
\(^2\)AAPS Journal 2007: 9 (1) Article 11
\(^3\)AAPS Journal 2007: 9 (3) Article 40
\(^4\)CVG White Paper from 2cnd Workshop on Recent Issues in GLP Bioanalysis, April 2008
SPE Extraction Procedure: Calculating Recovery

Blank Sample Matrix
(No analyte(s))

Spike Standards into Blank Matrix

Sample Matrix
(with analyte(s))

Spike Standards into Extracted Matrix

Extracted Sample
(with analyte(s))

Post-Extracted SPIKED Sample

% RE = \( 100 \times \frac{\text{RESPONSE Extracted Sample (with analyte(s))}}{\text{RESPONSE Post-Extracted SPIKED Sample}} \)

Both extracted samples should be in the same solution

Recovery of the Extraction Procedure (RE)*
(or, SPE Recovery)

Quantitative Assessment of Matrix Effects: Post-Extracted Spiked Sample

Blank Sample Matrix (No analyte(s))

Standard Solution (Analyte(s))

Matrix Factor (MF) = \frac{\text{Response}_{\text{Presence of Matrix}}}{\text{Response}_{\text{Absence of Matrix}}}

% Matrix Effects (ME) = \left( \frac{\text{Response}_{\text{Post-Extracted Spiked Sample}}}{\text{Response}_{\text{Solvent Standard}}} - 1 \right) \times 100

- Both samples should be in the same composition solution
- MF Value < 1, negative % ME = suppression
- MF Value > 1, positive % ME = enhancement