Challenges of validating small molecule LC-MS/MS biomarker methods
Introduction

- Bioanalytical classification
- White paper guidance
- Risk based approach to qualification/validation
- Setting acceptance criteria
- Endogenous assay validation – specific issues
- Surrogate matrix calibration
- Case study

Illustrated with answers from recent GCC questionnaire
Does your organisation apply different classification to biomarkers based on study endpoint and what are they?

- Primary Differentiation for Bioanalysis:- Exploratory versus Study-endpoint

No
Yes
Validation vs Qualification – Risk Approach

Biomarker Assays—Fit for Purpose

Pre-clinical Target Identification

Phase 0
Are we hitting the desired target in vitro?

Phase 1
Any hope of hitting the target in vivo?

Phase 2
Any indication of activity?

Phase 3
Surrogate for clinical outcome?

Approval?

Regulatory Risk (…yours too)

Your risk (not so much regulatory)

Brian Booth - Reid Bioanalytical Forum July 2009
GCC Questionnaire – Method Qualification

When would your organization apply a “fit-for-purpose” qualification to a biomarker method?

- Regulated method not achievable
- Everything
- At Sponsor's request
- Exploratory Biomarkers
What parameters would you include in a “fit-for-purpose” qualification of a biomarker

• Small molecule (/10 replies)
  – Calibration (10)
  – P&A (10)
  – Selectivity (9)
  – Matrix Effects (7), Parallelism (3)
  – Storage stability (7)
  – Sensitivity (6)
    – Linearity of dilution (4)
    – Recovery (4)
    – Others (reference ranges, carry-over)
What industry reference documents do you refer to for biomarker “qualification/validation”?

- FDA/EMA guidance
- Lee et al. (2006) & (2009)
- Chau et al. (2008)
- Cummings et al. (2010)
- Valentin et al. (2011)
- CLSI guidelines (formerly NCCLS)
Fit-for-Purpose Validation – Flow Chart

• Establish expectations of sponsor or scientific goal

• Define the purpose of the assay in terms of target values and acceptance limits

• Characterise performance of method by experimentation

Do you set acceptance criteria before or after the method “qualification/validation” for biomarker methods?

- ICON – Acceptance criteria for QCs during sample analysis is statistically linked to the performance of the method at validation using a confidence limit approach.
Endogenous Assay Validation – Specific Issues

• The issue of endogenous assay validation is not well described in the regulations for small molecules
• Different approaches include:
  – surrogate analyte
  – standard addition and extrapolation
  – surrogate matrix
• Choice of surrogate matrix
  – analyte free (hooray!)
  – stripped
  – synthetic

Beware matrix effects!
For small molecule biomarker methods, do you use a surrogate matrix (SM), standard addition (SA) or some other approach (other)?
Standard Addition Calibration

Extrapolation! Spiked Concentration

Response
Standard Addition Calibration

• Advantages
  – Matrix match calibration stds and samples
• Disadvantages
  – Difficult to estimate the LLOQ
  – Quantification software not always designed to handle standard addition calibration
  – Difficult to construct standard addition calibration where endogenous concentrations are high
  – bioanalytical regulations discourage extrapolation of calibration
Surrogate Matrix Calibration

• Advantages
  – Conventional quantitative processing of calibration
  – LLOQ instrument response can be measured directly (albeit in surrogate matrix)
  – No extrapolation of calibration

• Disadvantages
  – High probability of matrix effects

Quotient approach is surrogate matrix calibration for small molecule LC-MS/MS applications
Method Development – Batch Design

- Calibrate in surrogate matrix
- Use mix of matrix, diluted matrix and surrogate matrix QCs
  - Medium QC (undiluted pooled control matrix)
  - High QC (spiked control matrix)
  - Low QC (diluted control matrix ~x3 LLOQ)
  - LLOQ QC (spiked surrogate matrix)
- Minimise any potential matrix effects during method development
- With LC-MS/MS, SIL IS greatly increases the chances of success
- Check %RE of diluted matrix during method development
Androstendione in human urine

<table>
<thead>
<tr>
<th>QC ID</th>
<th>LLOQ 0.200 ng/mL</th>
<th>QC LOW 0.496 ng/mL</th>
<th>QC MED 12.4 ng/mL</th>
<th>QC HIGH 132 ng/mL</th>
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<tbody>
<tr>
<td></td>
<td>Surrogate Matrix</td>
<td>Diluted Matrix</td>
<td>Matrix</td>
<td>Spiked Matrix</td>
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<tr>
<td>Replicate 1</td>
<td>0.218</td>
<td>0.516</td>
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<td>5</td>
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<td>~0.620</td>
<td>12.1</td>
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<td>7</td>
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<td>10</td>
<td></td>
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<td>Intrarun SD</td>
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<td>12.3</td>
<td>0</td>
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<tr>
<td>n</td>
<td>5</td>
<td>6</td>
<td>10</td>
<td>6</td>
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</table>

Measured endogenous concentration in control urine

Intrarun %CV and Intrarun %RE values indicate variability and bias in the assay. A bias would probably indicate uncorrected matrix effects or differential recovery.
Method Validation - Parallelism

Examine diluted matrix n=6

• Fit--for-purpose method development and validation for successful biomarker measurement. J. W. Lee et al., Pharm. Res. 23(2):312-328 (2006).
# Method Validation – Matrix Effects

## Etiocholanolone in urine

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Undiluted Matrix</th>
<th>Diluted Matrix (1:5)</th>
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<tbody>
<tr>
<td></td>
<td>Mean n=6 (ng/mL)</td>
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<tr>
<td>Control 1</td>
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<td>Control 2</td>
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<td>Control 3</td>
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<td>Control 4</td>
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<tr>
<td>Control 5</td>
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<td>36.6</td>
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<tr>
<td>Control 6</td>
<td>2493.3</td>
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## Method Validation – Matrix Effects

### Androsterone in urine

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<tbody>
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<td></td>
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<td>107.0</td>
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<td>5435.0</td>
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<td>Control 4</td>
<td>591.7</td>
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<tr>
<td>Control 5</td>
<td>1153.3</td>
<td>41.8</td>
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<tr>
<td>Control 6</td>
<td>892.7</td>
<td>21.6</td>
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Application of Surrogate Matrix Approach - Cortisol Metabolism

Cortisol → 5β-Reductase → Dihydrocortisol → 3α-HSD → Tetrahydrocortisol

Cortisol → 11β-HSD → Cortisone → 5β-Reductase → Dihydrocortisone → 3α-HSD → Tetrahydrocortisone
Steroid Ratio 1

Steroid Ratio =

Indicative of 11β-HSD1 enzyme inhibition
### QC High

<table>
<thead>
<tr>
<th></th>
<th>5a-THF</th>
<th>a-cortol</th>
<th>a-cortolone</th>
<th>b-cortol</th>
<th>b-cortolone</th>
<th>THE</th>
<th>THF</th>
<th>Ratio 1</th>
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<tr>
<td>Mean</td>
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<td>1215</td>
<td>5273</td>
<td>1320</td>
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<td>16831</td>
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### QC Low

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<tr>
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<th>5a-THF</th>
<th>a-cortol</th>
<th>a-cortolone</th>
<th>b-cortol</th>
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<th>THE</th>
<th>THF</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
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<tbody>
<tr>
<td>ng/mL</td>
<td>324</td>
<td>32</td>
<td>254</td>
<td>53</td>
<td>188</td>
<td>1230</td>
<td>436</td>
<td>0.505</td>
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<tr>
<td>Mean</td>
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<td>31</td>
<td>245</td>
<td>45</td>
<td>188</td>
<td>1141</td>
<td>424</td>
<td>0.51</td>
<td>0.64</td>
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<td>SD</td>
<td>41</td>
<td>4</td>
<td>29</td>
<td>7</td>
<td>22</td>
<td>134</td>
<td>54</td>
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<td>0.03</td>
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<td>%CV</td>
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<td>11.8</td>
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<tr>
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<td>-3.5</td>
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### QC Medium

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<th>a-cortolone</th>
<th>b-cortol</th>
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<th>THE</th>
<th>THF</th>
<th>Ratio 1</th>
<th>Ratio 2</th>
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</thead>
<tbody>
<tr>
<td>ng/mL</td>
<td>1620</td>
<td>160</td>
<td>1270</td>
<td>265</td>
<td>938</td>
<td>6140</td>
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<td>155</td>
<td>1206</td>
<td>254</td>
<td>913</td>
<td>6072</td>
<td>2131</td>
<td>0.49</td>
<td>0.60</td>
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<tr>
<td>SD</td>
<td>149</td>
<td>13</td>
<td>109</td>
<td>25</td>
<td>87</td>
<td>498</td>
<td>196</td>
<td>0.02</td>
<td>0.03</td>
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<tr>
<td>%CV</td>
<td>9.7</td>
<td>8.4</td>
<td>9.0</td>
<td>10.0</td>
<td>9.6</td>
<td>8.2</td>
<td>9.2</td>
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<tr>
<td>%RE</td>
<td>-5.5</td>
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<td>-1.1</td>
<td>-2.3</td>
<td>-2.5</td>
<td>-3.6</td>
</tr>
</tbody>
</table>

>23% QC failure at +/-15%

<0.5% QC failure at +/-15%

<12% QC failure at +/-20%
SAD Data with Confidence Limits

**Dose 1**

- Steroid Ratio vs Time (hrs)
- Ave - dosed and Ave - placebo

**Dose 4**

- Steroid Ratio vs Time (hrs)
- Ave - dosed and Ave - placebo

**Dose 2**

- Steroid Ratio vs Time (hrs)
- Ave - dosed and Ave - placebo

**Dose 7**

- Steroid Ratio vs Time (hrs)
- Ave - dosed and Ave - placebo
Generic Approach

Surrogate matrix calibration

Generic Approach

Use of SIL IS

Applied to both up/down regulation

Track record of use

Fit-for-purpose validation

Applied to wide range of small molecule endogenous analytes
Summary

• Bioanalytical classification of biomarkers; exploratory versus study endpoint
• Use risk based approach to determine extent of qualification/validation
• Opportunity to introduce biomarkers earlier (preclinical) at less cost
• Minimum for a qualification should probably include - Calibration, P&A, Selectivity, Matrix effect, Stability (limited) and Sensitivity
• Consider setting acceptance criteria based on performance of the assay during validation
• Use of surrogate matrix offers a relatively simple generic approach