Hemolyzed Sample Evaluation

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Hemolyzed Sample Evaluation

- Brief Description
- Industry Perspective
- Hemolyzed Sample Preparation
- Validation of Samples
- Primary Causes of Failures
- Sample Analysis Verification
- Example Data
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- Acknowledgement
Description of Hemolyzed Samples

- Hemolysis:
  - The destruction of red blood cells which leads to the release of hemoglobin from within the red blood cells into the blood plasma

- Causes
  1. May occur *in vivo* as a result of pathology or drug effect
  2. May occur during the collection of blood
  3. May occur during the processing of collected blood into plasma/serum
Industry Perspective

- Hemolysis has been discussed for many years.
- Recently, FDA conference white papers and draft EMEA guidelines are calling for the assessment of hemolyzed samples during validations.
- Testing should occur as part of the matrix effect evaluation.
Hemolyzed Sample Preparation

- Sample preparation questions:
  1. Typically, add small percentage of blood to plasma samples
     - Does this represent “hemolyzed samples”
     - Is there a better way to prepare hemolyzed samples?
  2. Sample Design
     - Multiple lots (matrix effect) – appears to be preferred method
     - Single lots (stability evaluation)
  3. Condition of blood
     1. Fresh blood – Allows blood cells to lyse in presence of drug
     2. Frozen - lysed blood provides immediate and consistent access of blood to blood cell contents – worst case scenario
Hemolyzed Sample Acceptance Criteria

1. Quantitation using non-hemolyzed standards
   - Does not test only the impact of hemolysis
   - This is the industry accepted criteria for most validation evaluations

2. Matrix factor
   - What result would indicate a failure
   - Results may have no well defined acceptance criteria

3. Quantitatively compare to control
   - Compare to sample without blood
   - Scientifically sound
Validation of Hemolyzed Samples

- Current Process
  1. Fresh or frozen blood may be added to plasma – mandatory 24-hour freeze cycle prior to analyzing
  2. Consultation with sponsors, other CROs led to using 2% (v/v) blood added to plasma/serum
  3. Hemolyzed samples analyzed at a minimum of 3 individual/pool lots at low and high QC concentration

- Acceptance criteria:
  - 2/3 of samples within +/- 15% of theoretical conc.
  - Inter-lot % bias and % CV less that 15%
Primary Causes of Failures

1. Matrix effect - Adjust method to eliminate effect
   - Extraction – Enhance sample clean-up using an SPE method instead of protein precipitation
   - Chromatography – run a gradient instead of isocratic
   - LBS – add in extraction or dilution

2. Stability - Change Conditions
   - Long-term Storage at -80°C instead of -20°C
   - Collection or processing conditions – use ice water bath instead of ambient temperature

3. Recovery - Adjust extraction conditions to improve recovery
   - Change pH, aqueous to organic ratio, SPE conditions, etc.
Primary Causes of Failures

4. Selectivity
   - Improve sample clean-up to remove interference
   - Dilute out interference
   - Separate interference chromatographically

5. Endogenous Content
   - High levels in red blood cells
   - Little chance to correct, adjust LLOQ above endogenous level

- Fixing the method is primary focus of hemolysis testing during Method Development
Sample Analysis Verification - Failure

- If the failure cannot be overcome:
  - Test range of percentages
    - Typically test 1, 2, and 5% (v/v)
  - Document Samples that are hemolyzed
    - All samples are noted as hemolyzed
    - Document percentage of hemolysis
  - Report samples that may be affected by high levels of hemolysis
Sample Analysis Verification - Failure

- Use color chart to evaluate samples:
  - 1% and 2% hemolysis can be differentiated
  - 3% – 5% difficult to differentiate
  - Above 5% very dark red cannot be differentiated
### Example #1

- Methanol precipitation – 100% recovery - plasma
- Methanol precipitation (MeOH) – 76% loss of recovery in hemolyzed samples
- Acidified methanol (MeOH + TCA) – 108% recovery in Hemolyzed Samples

<table>
<thead>
<tr>
<th></th>
<th>Control QC</th>
<th>Hemolyzed QC</th>
<th>Hemolyzed QC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MeOH</td>
<td>MeOH + TCA</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>1161440</td>
<td>256911</td>
<td>1196435</td>
</tr>
<tr>
<td>MeOH + TCA</td>
<td>1085956</td>
<td>296718</td>
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<td>NV</td>
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<tr>
<td>Mean</td>
<td>1123698</td>
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<tr>
<td>% CV</td>
<td>4.8</td>
<td>7.6</td>
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<tr>
<td>% Control</td>
<td>24</td>
<td>108</td>
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<tr>
<td>n =</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

MeOH = Precipitation method using only methanol  
MeOH + TCA = Precipitation using TCA and methanol
### Example #2

**EDTA plasma**
- No recovery in hemolyzed plasma samples with methanol only extraction
- 109% recovery in hemolyzed plasma samples using methanol + TCA extraction

<table>
<thead>
<tr>
<th></th>
<th>MeOH</th>
<th>TCA + MeOH</th>
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<tbody>
<tr>
<td></td>
<td>QC</td>
<td>REC</td>
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<tr>
<td><strong>Mean</strong></td>
<td>0</td>
<td>1132</td>
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<tr>
<td><strong>C.V. %</strong></td>
<td>na</td>
<td>7.1</td>
</tr>
<tr>
<td><strong>% Recovery</strong></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**QC** = Quality Control with compound spiked prior to extraction  
**REC** = Blank plasma sample spiked with analyte after extraction
Example #2

- **Heparin plasma**
  - 101% recovery from hemolyzed samples with methanol only extraction
  - 99% recovery from hemolyzed samples with methanol + TCA Extraction

<table>
<thead>
<tr>
<th></th>
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<th>TCA + MeOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QC</td>
<td>REC</td>
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<tr>
<td><strong>Mean</strong></td>
<td>1276</td>
<td>1264</td>
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<tr>
<td><strong>C.V. %</strong></td>
<td>7.0</td>
<td>1.3</td>
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<tr>
<td><strong>% Recovery</strong></td>
<td>101</td>
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</table>

**QC** = Quality Control with compound spiked prior to extraction

**REC** = Blank plasma sample spiked with analyte after extraction
Example #3

- Initial failure at 2% base on +/- 15% acceptance criteria
- Evaluated range of percentages
- 1% passes and 2% fails
- Samples reported with greater than 1% hemolysis would be deemed questionable

<table>
<thead>
<tr>
<th>Percent Hemolyzed</th>
<th>Low % Bias</th>
<th>High % Bias</th>
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<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>-1</td>
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<tr>
<td>0.5</td>
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</tr>
<tr>
<td>1</td>
<td>1</td>
<td>-14</td>
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<tr>
<td>2</td>
<td>-13</td>
<td>-16</td>
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<tr>
<td>5</td>
<td>-22</td>
<td>-12</td>
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Hemolyzed Sample – Conclusions

- Evaluate hemolyzed samples early in method development to avoid re-development.
- If a failure occurs in hemolyzed samples, evaluate reason for failure to determine best course of action.
  - If endogenous compound is being tested, resolution of issues raised by hemolysis may be difficult.
- Eliminate subjectivity as much as possible.
- Well-trained scientists is key to hemolysis evaluations.
- SOPs that:
  1. Specify how to prepare and test samples.
  2. How to address hemolyzed clinical/pre-clinical samples.
Acknowledgements

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