Hemolyzed Sample Evaluation

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Hemolyzed Sample Evaluation

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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 hemolzed 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

- 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

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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**

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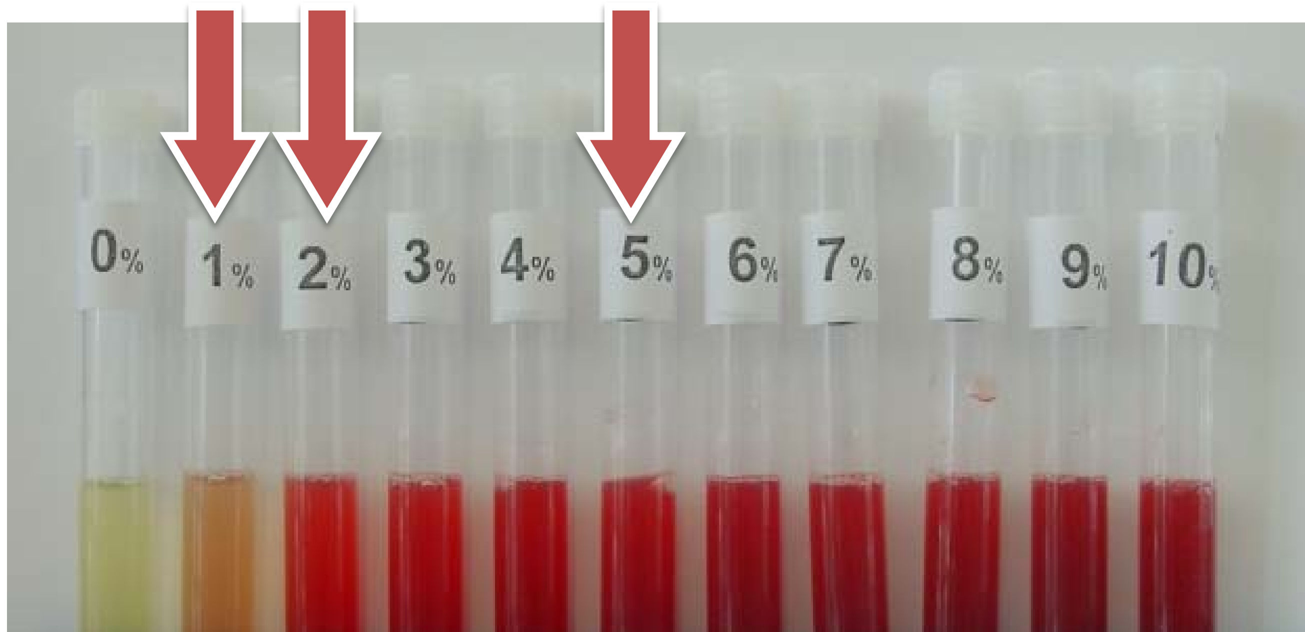
Sample Analysis Verification - Failure

- 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

	Control QC	Hemolyzed QC MeOH	Hemolyzed QC MeOH + TCA
	1161440	256911	1196435
	1085956	296718	1226431
	NV	266695	1223119
Mean	1123698	273441	1215328
% CV	4.8	7.6	1.4
% Control		24	108
n =	2	3	3

MeOH = Precipitation method using only methanol
MeOH + TCA = Precipitation using TCA and methanol

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

Example #2

Hemolyzed EDTA Plasma				
	MeOH		TCA + MeOH	
	QC	REC	QC	REC
Mean	0	1132	2780	2539
C.V. %	na	7.1	13.5	7.5
% Recovery		0		109
QC = Quality Control with compound spiked prior to extraction REC = Blank plasma sample spiked with analyte after extration				

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

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Example #2

Hemolyzed Heparin Plasma				
	MeOH		TCA + MeOH	
	QC	REC	QC	REC
Mean	1276	1264	3036	3064
C.V. %	7.0	1.3	6.2	17.8
% Recovery		**101**		**99**
QC = Quality Control with compound spiked prior to extraction REC = Blank plasma sample spiked with analyte after extration				

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

Example #3

Percent Hemolyzed	Low % Bias	High % Bias
0	2	-1
0.5	0	2
1	1	-14
2	-13	-16
5	-22	-12

- 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

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

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