



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 and other proteins 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

- The impact of hemolysis on bioanalysis 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
- Should occur as part of the matrix effect evaluation during method validation

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

- 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 than 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

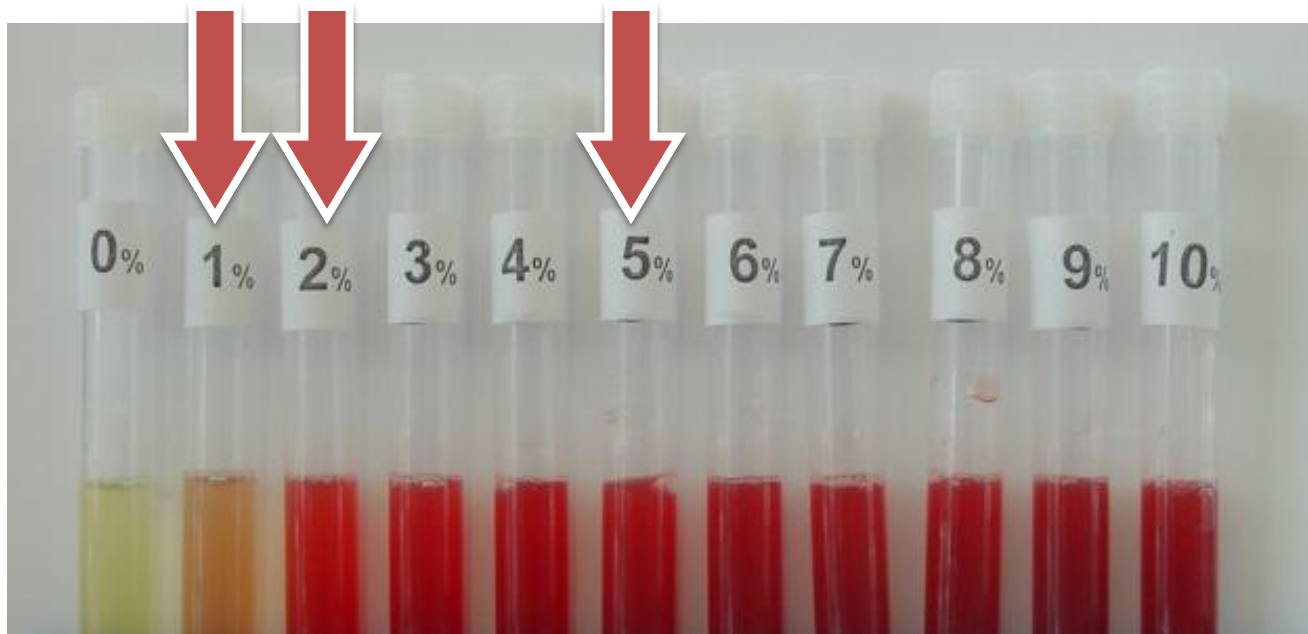
- High levels in red blood cells
- Little chance to correct, adjust LLOQ above endogenous level

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 visually 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 extraction

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

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 extraction

- 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 are 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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