CASE STUDIES IN HAEMOLYSED PLASMA ASSESSMENT FOR BIOANALYTICAL METHOD VALIDATION USING LC-MS/MS

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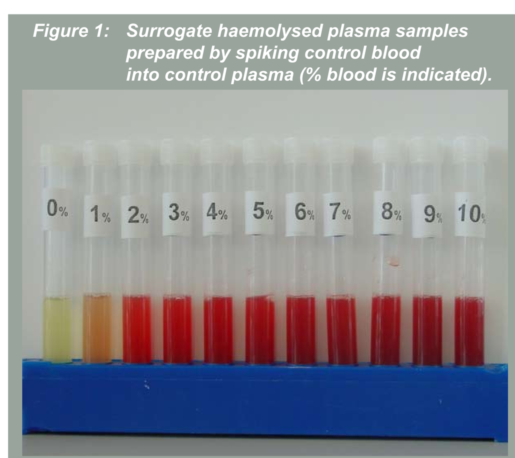
INTRODUCTION

The evaluation of the impact of sample haemolysis during validation of bioanalytical methods used to determine drugs and metabolites in plasma matrices has recently become part of the regulatory guidances.
Haemolysis describes the process of rupturing erythrocytes whereby releasing their cellular components into the plasma fraction. This process can occur in vivo as a result of a diseased state or dosed drug or in vitro during the blood/plasma collection process.
This presentation describes the current global validation procedure at Celerion for assessment of the effect of haemolysis and cites case study examples when haemolysis has shown to have an effect on analyte quantitation. These include effects on ionisation modification, analyte stability, recovery and method selectivity. In such cases the reasons and symptoms of effect are examined and the strategies for dealing with such occurences either in validation or sample analysis studies are discussed.

HAEMOLYSED PLASMA ASSESSMENT PROCEDURE

Recent bioanalytical method validation guidance changes have recommended the assessment of effect of haemolysis (as part of the overall matrix effect evaluation). The procedure used at Celerion performs haemolysis evaluation in line with existing matrix effect evaluations during the method development and method validation phases of a bioanalytical method.
Following discussion as to how to best and consistently represent haemolytic samples initial evaluations were performed using surrogate haemolysed plasma prepared by spiking blood containing identical anticoagulant into control plasma at 5% by volume. This has been subsequently amended to 2% by volume to be more representative of real haemolysed samples (Figure 1). This surrogate haemolysed matrix may be prepared freshly or frozen prior to analyte spiking. Spiked samples are subjected to a single freeze-thaw cycle prior to processing.
At least 3 such haemolysed plasma lots (previously a single lot) are now tested under our procedure (at blank, low and high QC levels). 2/3 of the haemolysed plasma lots at each spiked level should be within normal QC validation and blank acceptance criteria for the haemolysed plasma assessment to be considered acceptable.

Figure 1: Surrogate haemolysed plasma samples prepared by spiking control blood into control plasma (% blood is indicated).



CASE STUDY 1 – MATRIX EFFECT

This case study illustrates an observed matrix effect difference between haemolysed and non-haemolysed plasma matrix resulting in potential over-estimation of analyte concentrations in haemolysed samples when compared to a control plasma standard curve.
An on-line extraction (turboflow) LC-MS/MS (-ve ESI) method for the determination of a small molecule was developed and validated. The method met all validation criteria with respect to sensitivity, precision and accuracy, selectivity and matrix effect in control plasma lots and analyte stability. Method quantitation was performed with an LLOQ of 5 ng/mL and utilised a structural analogue internal standard (Figure 2).
Analysis during the method development phase of fortified frozen and thawed quality control samples prepared in surrogate haemolysed plasma (blood containing the same anticoagulant spiked into plasma at 5% volume) initially showed measured concentrations with a +20-40% bias. This observation was further investigated and the root cause isolated to a time dependant ionisation enhancement effect for the analyte (not tracked by the analogue internal standard). This effect was only seen in surrogate haemolysed plasma samples stored under autosampler storage conditions (refrigerated nom +5°C) for more than 14 hours. Post column infusion experiments showed clearly the ionisation enhancement effect (Figure 3). This effect was reproduced on several occasions and with different matrix lots. A pure blank selectivity effect was ruled out. In all cases the chromatographically resolved internal standard was unaffected and whilst the magnitude of enhancement for the analyte was small it was sufficient to produce a recognised bias outside of acceptance criteria for surrogate haemolysed samples (Table 1).

Figure 2: Example chromatogram showing retention of analyte and internal standard respectively (partial resolution)

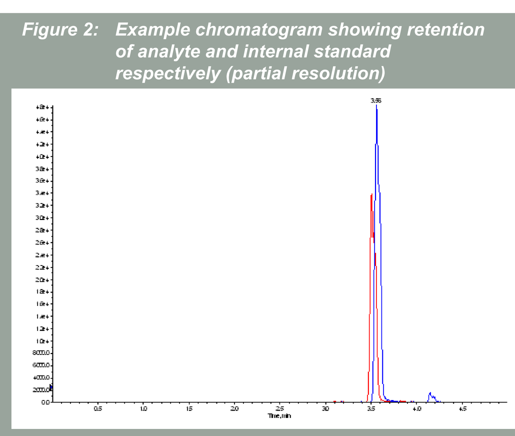


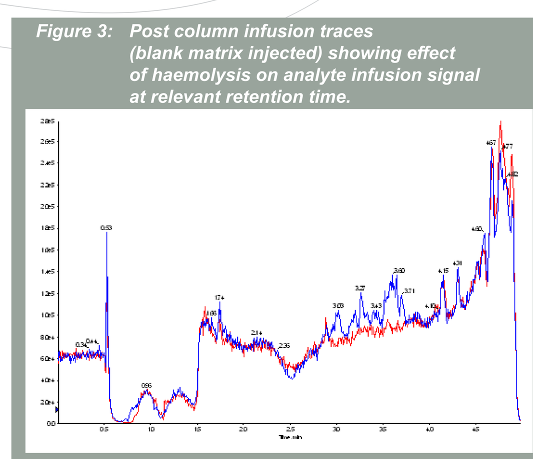
Table 1: Accuracy and precision of haemolytic and non-haemolytic QC samples (frozen)

QC LEVEL	LLOQ		HIGH	
Nom (ng/mL)	5	%	1600	%
	4.60	92.0	1480	92.5
Control	4.65	93.0	1570	98.1
human plasma	4.92	98.4	1510	94.4
	5.00	100	1520	95.0
	4.62	92.4	1580	98.8
	4.58	91.6	1750	109.4
Mean	4.73		1568	
% CV	3.9		6.2	
% Bias	-5.4		-2.0	
n	6		6	

QC LEVEL	LLOQ		HIGH	
Nom (ng/mL)	5	%	1600	%
	(10.9)*	218.00	2030	126.9
Haemolysed	(3.90)*	78	2010	125.6
human plasma	6.37	127.4	2000	125.0
	7.28	145.6	2080	130.0
	7.05	141	2070	129.4
	7.12	142.4	1970	123.1
Mean	6.96		2027	
% CV	5.8		2.1	
% Bias	39.1		26.7	
n	4		6	

* = unacceptable chromatography, removed from calculations

Figure 3: Post column infusion traces (blank matrix injected) showing effect of haemolysis on analyte infusion signal at relevant retention time.



Whilst the set of circumstances leading to the observed effect may be unique (on-line extraction therefore blood cell components are present in the sample for an extended period plus a non co-eluting internal standard) it does illustrate a real and reproducible effect. The problem could be eliminated by methodology changes including:

- Changing to an alternative off-line extraction.
- Chromatographic modification to force co-elution of analyte with internal standard.
- Changing MS/MS ESI polarity (the overall effect was demonstrated to be much less significant in +ve ESI).

CASE STUDY 2 – STABILITY EFFECT

This case study illustrates an observed stability effect difference between haemolysed and non-haemolysed plasma matrix after multiple freeze and thaw (FT) cycles. An automated liquid-liquid extraction LC-MS/MS (+ve ESI) method for the determination of a small molecule was developed and validated. The method met all validation criteria with respect to sensitivity, precision and accuracy, selectivity, matrix effect and stability in control plasma lots.

Analysis during the method development phase of fortified frozen (-20°C) and thawed (on ice water) integrity samples prepared in surrogate haemolysed plasma (whole blood containing the same anticoagulant spiked into plasma at 5% v/v) initially showed measured concentrations within 10% bias for both x1 and x3 FT cycles (See Table 2). After 6 repeated FT cycles the quantitation was approximately 16% low biased. The control QCs when cycled similarly to the haemolysed samples did not show the same trend with all samples showing less than 4% bias for up to 6 FT cycles.

Table 2: Precision and accuracy of 3 lots of haemolysed plasma versus number of FT cycles

	LLOQ (1.00 ng/mL)			
	Lot#	x1 FT	x3 FT	x6 FT
	1	1.00	1.00	0.906
	2	0.853	0.840	0.784
	3	0.871	0.886	0.830
Mean		0.907	0.909	0.840
% CV		8.7	9.1	7.3
% Bias		-9.3	-9.1	-16.0
n		3	3	3
Stability		PASS	PASS	FAIL

Whole blood stability of the analyte was evaluated at ambient temperature and indicated the analyte to be unstable. The collection evaluation was repeated with the blood being kept on an ice water bath resulting in acceptable stability for up to 2 hours. From this test it was hypothesized that some components of whole blood used to fortify the haemolysed surrogate matrix could lead to degradation of the analyte.

The above case study is an exemplification of where control matrix and possible real-world samples may not have similar behaviours with respect to stability and thus should be investigated.

CASE STUDY 3 – RECOVERY

This case study illustrates an observed impact on the recovery of an analyte due to the presence of small quantities of whole blood present in the plasma samples. Further testing of the haemolysed samples also illustrated a plasma anti-coagulant dependent effect on recovery due to haemolysis.

The original method developed was a protein precipitation method using methanol. This method provided acceptable results against validation criteria in multiple lots of EDTA plasma. When the method was tested using haemolysed samples (2% blood by volume), recovery decreased by nearly 75% (Table 3).
Modification of the extraction method showed that the addition of acid corrected the recovery issue. Multiple acids were evaluated at different strengths. Although many acid types and strengths produce acceptable recovery, TCA was chosen due to optimal precision (Table 3). The final method used an addition of TCA followed by methanol.

Table 3: Comparison of recovery loss in haemolysed samples. Data shown is for a high concentration QC sample in control plasma using original method, haemolysed sample with original method, and haemolysed sample using acidic extraction method.

QC LEVEL (Precipitant)	HIGH (MeOH)	HAEM HIGH (MeOH)	HAEM HIGH (TCA+MeOH)
	1161440	256911	1196435
Area counts	1085956	296718	1226431
		266695	1223119
Mean	1123698	273441	1215328
% CV	4	7.6	1.4
% Control	100	24.3	108
n	2	3	3

An comparison of haemolysed EDTA plasma versus haemolysed heparinised plasma was also performed. Recovery testing indicated that the haemolysis effect was isolated to EDTA plasma, and that heparin plasma did not show the same issue (Table 4).

Table 4: Comparison of haemolysis effect in EDTA and heparinised plasma. Data shown is a low concentration QC in haemolysed plasma using original method with no acid in the extraction versus the revised method using acidic protein precipitation method.

Heparin	MeOH		TCA + MeOH	
	QC	REC	QC	REC
	1224	1274	2907	3685
Area counts	1379	1245	2950	2846
	1224	1272	3251	2660
Mean	1276	1264	3036	3064
% Recovery	101		99	

EDTA	MeOH		TCA + MeOH	
	QC	REC	QC	REC
	0	1051	2540	-
Area counts	0	1213	2587	2673
	0	1133	3212	5405
Mean	0	1132	2780	2539
% Recovery	0		109	

QC = Quality control sample spiked with analyte prior to extraction

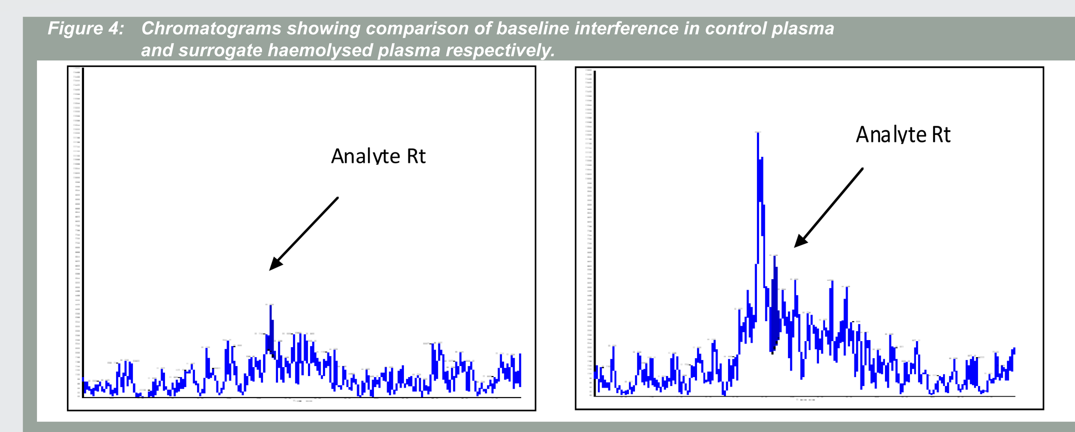
REC = Blank plasma sample spiked with analyte post extraction

This evaluation suggests that early testing of recovery in haemolysed matrix lots should be considered to ensure efficiency in bioanalytical method development. A simple change in pH of the extraction conditions altered the recovery of this analyte by over 75%.

CASE STUDY 4 - SELECTIVITY

This case study illustrates an observed selectivity effect difference between haemolysed and non-haemolysed plasma matrix resulting in imprecision and inaccuracy of analyte concentrations in haemolysed samples when compared to a control plasma standard curve.
An LC-MS/MS (+ve ESI) method following protein precipitation for the determination of a therapeutic peptide was developed and validated. The method met all validation criteria with respect to sensitivity, precision and accuracy, selectivity and matrix effect in control plasma lots, analyte stability. Method quantitation was performed with an LLOQ of 1.94 nM and utilised a structural analogue internal standard.
Analysis during the method development phase of fortified frozen and thawed quality control samples prepared in surrogate haemolysed plasma (blood containing the same anticoagulant spiked into plasma at 5% volume) showed measured concentrations at the LLOQ level with a +30-40% bias and a precision of > 20% due to a consistent interference at the retention time of analye in surrogate haemolysed plasma (Figure 4).

Figure 4: Chromatograms showing comparison of baseline interference in control plasma and surrogate haemolysed plasma respectively.



The impact of haemolysis on accuracy and precision is demonstrated in the following table (Table 5). The method allowed for accurate and precise determination at the Low QC level (5.83nM), below this level data was regarded as not reportable.

Table 5: Accuracy and precision of haemolytic and non-haemolytic QC samples (frozen).

QC LEVEL	Human Plasma		Haemolysed Plasma	
(ng/mL)	LLOQ	LOW	LLOQ	LOW
	2.43	5.61	2.54	5.44
Measured conc	2.37	6.29	2.81	5.53
(nM)	2.06	6.05	BLQ	5.79
	2.33	5.70	2.46	6.31
	2.18	4.78	3.92	5.32
	2.23	5.08	BLQ	6.10
Mean	2.26	5.58	2.60	5.75
% CV	6.0	10.2	28.1	6.8
% Bias	16.7	-4.2	34.1	-1.4
n	6	6	6	6

CONCLUSION

This presentation illustrates the array of potential effects haemolytic plasma samples can generate on the performance of a bioanalytical method. Whilst the procedure used for assessment is not ideal and only provides at best a surrogate matrix for testing, it does provide consistency between and within assay evaluations. Celerion is currently performing comparison tests against alternative sources of haemolysed plasma including commercially available sources. This may however bring additional issues of characterisation and consistency.
On encountering such effects in haemolytic plasma the primary question must always be how to proceed and this must be dealt with on a case by case basis. The effect may be eliminated by redevelopment of the method or by diluting out the effect with control plasma. In the event that both of these approaches are unsuccessful the only option remains to identify all haemolysed study samples and exclude from analysis and reporting.

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