INTRODUCTION

The evaluation of the impact of sample haemolysis during validation of bioanalytical methods is dependent on detection limits and metabolite plasma concentrations. Recent publications have reported that metabolite plasma concentrations in haemolysed samples have become part of the regulatory guidelines. Haemolysed plasma is used to test bioanalytical methods to determine if they are adequate for use in clinical studies. This paper describes the results of an internal validation procedure at Celerion for assessment of the effect of haemolysis and cites case study examples when haemolysed plasma has shown to have an effect on analyte quantitation. These include effects on ionisation modification, analyte stability, and method selectivity. In such cases the reasons and symptoms of effect are examined and the strategies for dealing with such occurrences either in validation or sample analysis studies are discussed.

HAEMOLYSED PLASMA ASSESSMENT PROCEDURE

Recent bioanalytical method validations in our lab have changed our approach to sample matrix effect evaluation in pharmacokinetic studies 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 represent haemolysed samples, initial matrix effect evaluations were performed using surrogate haemolysed plasma prepared by freezing 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).

The surrogate haemolysed matrix was defined by the analysis of a plasma matrix prepared by spiking control blood with analyte and internal standard (Figure 2). The surrogate haemolysed plasma 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 surrogate haemolysed plasma lots (previously a single lot) are now tested under our protocol (at bland, 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 matrix to be considered acceptable.

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 (bubbling) LC-MS/MS (re LCMS) method for the determination of a small molecule was developed and validated. The method met all validation criteria with respect to selectivity, precision, accuracy, sensitivity and matrix effect in control plasma lots and analyte stability. Method quantitation was performed with an LLOQ of 5 nM, and utilised a structural analogue internal standard (Figure 2).

Analyte recovery in fortified control plasma lots (blood containing the same anticoagulant spiked into plasma at 5% volume) was found to be 90% at 2% haemolysis, and showed 80% at 1% haemolysis using a structural analogue internal standard.

The method was subsequently used for analysis of fortified plasma. Whole blood stability of the analyte was evaluated at ambient temperature and found to have an acceptable shelf-life. The collection evaluation was performed on whole blood samples, and showed that the analyte had a detectable ionisation enhancement effect for the analyte (not tracked by the analogue internal standard). The effect was only seen in surrogate plasma haeomolysed with EDTA and not MeOH.

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

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 (re LCMS) method for the determination of a small molecule was developed and validated. The method met all validation criteria with respect to sensitivity, precision, accuracy, selectivity, matrix effect and stability in control plasma lots.

Analysis during the method development phase of fortified frozen (-20°C) and thawed (on-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 x2 FT cycles (Table 2). After 6 repeated FT cycles the quantitation was approximately 15% low biased. The control QC when cycled similarly to the haemolysed samples did not show the same trend with all samples showing less than 4% bias for up to 8 FT cycles.

CASE STUDY 3 – RECOVERY

This case study illustrates an observed impact on the recovery of an analyte due to the presence of small amounts 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 human plasma (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 concentrations. Although many acids at lower strength showed an increase in recoveries, recovery was not optimal. TCA was chosen due to optimal precision (Table 3). The final method used an addition of TCA followed by methanol.

CASE STUDY 4 – SELECTIVITY

This case study illustrates an observed selectivity effect difference between haemolysed and non-haemolysed plasma matrix resulting in precision and accuracy of analyte concentrations in haemolysed samples when compared to a control plasma standard curve.

An LC-MS/MS (re LCMS) 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, analytic 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 +50% bias and a precision of > 20% due to a consistent interference at the retention time of analyte in surrogate haemolysed plasma (Figure 4).

Table 1: Comparison of haemolysis effect in EDTA and heparinised plasma. Data shown is for a high concentration of analyte (% control is indicated).

<table>
<thead>
<tr>
<th>QC LEVEL</th>
<th>EDTA MeOH</th>
<th>TCA + MeOH</th>
<th>EDTA Heparin MeOH</th>
<th>TCA + MeOH Heparin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00</td>
<td>1.00</td>
<td>0.906</td>
<td>0.906</td>
</tr>
<tr>
<td>% COV</td>
<td>0.9</td>
<td>0.9</td>
<td>0.906</td>
<td>0.906</td>
</tr>
<tr>
<td>CV%</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

The impact of haemolysis on accuracy and precision is demonstrated in the following table (Table 4). The method allowed for accurate and precise determination at the Low QC level (8,383 nM), below this level data was regarded as not reportable.

Table 2: Comparison of haemolysis effect in surrogate human plasma samples.

<table>
<thead>
<tr>
<th>Lot #</th>
<th>x1 FT</th>
<th>x3 FT</th>
<th>x6 FT</th>
<th>x1 FT</th>
<th>x3 FT</th>
<th>x6 FT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.00</td>
<td>1.00</td>
<td>0.906</td>
<td>0.906</td>
<td>0.906</td>
<td>0.906</td>
</tr>
<tr>
<td>% COV</td>
<td>0.9</td>
<td>0.9</td>
<td>0.906</td>
<td>0.906</td>
<td>0.906</td>
<td>0.906</td>
</tr>
<tr>
<td>CV%</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
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</tr>
</tbody>
</table>

CONCLUSION

This presentation illustrates the array of potential effects haemolysed 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 additional 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 haemolysed plasma the primary question must always be how to proceed and this must be dealt with on a case by case basis. The selection of the appropriate method may be achieved by diluting out the effect with control plasma. In the event that both of these approaches are not possible, additional tests may be required to identify all haemolysed study samples and exclude from analysis reporting.

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