Development of an Intact C-peptide Liquid chromatography Tandem Mass Spectrometry Method for Serum/Plasma and Dry Blood Spot Samples.

KEYWORD: LCMS/MS, DBS, C-peptide, Diabetes, Dry Blood Spot, Mass Spectrometry, Liquid Chromatography, Insulin

INTRODUCTION

C-peptide is a by-product of insulin biosynthesis from the precursor, proinsulin (figure 1.0). Produced in equimolar quantities as insulin, C-peptide is used as a surrogate marker for insulin secretion. Laboratory estimation is used in the classification and treatment of diabetes as well as investigations into hypoglycaemia. Research studies and clinical trials use C-peptide levels to determine the effects of interventions designed to preserve and improve residual β-cell function. In practice, C-peptide is measured mostly by immunoassay methods using a random urine collection sample or venous blood. The limitations of C-peptide immunoassay include interference with a high dose of biotin as well as cross-reactivity with proinsulin which reduces specificity. Serum/plasma samples require immediate centrifugation and freezing which limits C-peptide measurement to healthcare settings equipped with suitable laboratories. Dried blood spot (DBS) microsampling is less invasive, simple, innovative alternative means of obtaining venous blood samples which could offer more stability. The combination of DBS and mass spectrometry offers improved sensitivity and selectivity. We have developed and validated an LC-MS/MS intact C-peptide method for serum/plasma samples that overcome the limitations of immunoassays. Taking advantage of the sensitivity offered by LCMS, we also investigate dry blood spot (DBS) samples for its performance consistency with appropriate clinically based performance goals. C-peptide measurement via LC-MS/MS will have an increased impact if DBS can be used as a sample of choice particularly in paediatrics and elderly patients.

MATERIALS AND METHODS

This method uses a simple protein precipitation coupled with solid phase extraction. DBS require an extra series of preparation steps, including punching 6mm discs from cards, elution using PBS/BSA before extraction following the same steps as serum/plasma. The LC separation was achieved using 0.1% Formic acid in water as mobile phase A and 0.1% Formic acid in Acetonitrile as mobile phase B. The column used was the Waters C18 10 μm. Analytes and internal standards were detected using the Waters Xevo TQ(S) in MRM ESI positive mode. The method was first validated for serum/plasma samples. An assessment of DBS specimen suitability was carried out following the CLSI guideline EP35-ED1 where the equivalence between serum/plasma and DBS specimen types was assessed in terms of systematic difference and imprecision at each medical decision level.

RESULTS

Serum/Plasma - Method Comparison

The comparison with Mercodia Elisa for serum/plasma shows a positive linear relationship and dispersion of results with an R² value of 1.0 and a slope of 0.9378 being achieved.

Serum/Plasma - Measurement Trueness

• The comparison with Mercodia Elisa for serum/plasma shows no significant difference from the ALT despite a slight negative bias.
• The z-scores were all within +/-2 which was acceptable.

Serum/Plasma - Linearity

• Linearity of the standard curve was demonstrated by the mean correlation coefficient values for 5 curves from separate runs. The mean R² values for C-peptide was greater than 0.99.
• The linearity using a high concentration patient sample demonstrated R² value of 0.9994.

CONCLUSIONS AND FUTURE WORK

In conclusion, an LCMS method has been developed and validated for the determination of C-peptide in serum/plasma and verified for dried blood spot using offline solid phase extraction. The method demonstrates good resolution of analyte peaks, with very little background noise on chromatograms, indicating an effective extraction process. The combination of protein precipitation followed by solid phase extraction and dilution of the final eluent was efficient with considerable removal of matrix effect. Mean recovery was greater than 95% and recovery was within ±15% of expected value. The measuring range of the assay was 3.9pmol/L to 8000pmol/L. This method will now be incorporated into the test results at Guiford peptides hormones laboratory for diabetic investigations. It will also be used to enhance the UKNEQAS Guildford peptides hormones EQA scheme and enable accuracy-based performance monitoring. An LCMS Assay using DBS sampling can support large-scale clinical trials in diabetes. Samples can be collected at home, thereby avoiding expensive and time consuming stimulated tests which are also difficult to replicate in clinics.

Future Work

Next stages of the research is DBS stability study and verification of DBS sampling against serum following the Clinical and Laboratory Standards Institute (CLSI) guideline EP35-ED1.

ACKNOWLEDGEMENT

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REFERENCES

3. CLSI. Assessment of Equivalence or Suitability of Specimen Types for Medical Laboratory Measurement Procedures EP35. 1st ed. Clinical and Laboratory Standards Institute; Wayne, PA. USA: 2019

Figure 1.0 Structure of proinsulin. It consist of Insulin A chain (red) and B chain (green) and the connecting peptide. C-peptide (blue) ensures alignment between the A and B chains.

Figure 2.0 Typical chromatogram for C-peptide showing quantitative m/z transitions . a. (Tyr⁰)-C-Peptide b. (Tyr⁰)-C-Peptide internal standard peak

Table 1.0 LC Conditions

<table>
<thead>
<tr>
<th>Gradient</th>
<th>Time (min)</th>
<th>Flow rate (μL/min)</th>
<th>% Mobile phase A</th>
<th>% Mobile phase B</th>
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</thead>
<tbody>
<tr>
<td>10%</td>
<td>0</td>
<td>0.250</td>
<td>85</td>
<td>15</td>
</tr>
<tr>
<td>65%</td>
<td>10</td>
<td>0.250</td>
<td>15</td>
<td>85</td>
</tr>
<tr>
<td>100%</td>
<td>20</td>
<td>0.250</td>
<td>15</td>
<td>85</td>
</tr>
</tbody>
</table>

Table 2.0 LC conditions

<table>
<thead>
<tr>
<th>Linear behaviour</th>
<th>Values (μL/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.000</td>
</tr>
<tr>
<td>Slope</td>
<td>0.9378</td>
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</tbody>
</table>

Figure 3.0 Patient sample bias plot showing the linear regression graph between the LCMS method and Mercodia ELISA kit.

Figure 4.0 Residuals and Difference plots for UK NEQAS Guildford peptide hormone scheme samples.

Figure 5.0 Representation of typical Calibration curve showing the correlation coefficient and slope.

Figure 6.0. Representation of linearity using high concentration patient sample.

Figure 7.0 Qualitative matrix effect chromatograms.

Figure 8.0 Chromatogram for C-peptide showing quantitative m/z transitions . a. (Tyr⁰)-C-Peptide b. (Tyr⁰)-C-Peptide internal standard peak