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

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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 uses C-peptide levels to determine the effects of interventions designed to preserve and improve residual β-cell function. In practise, 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 offers 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) as sample of choice, verifying that performance is consistent 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.

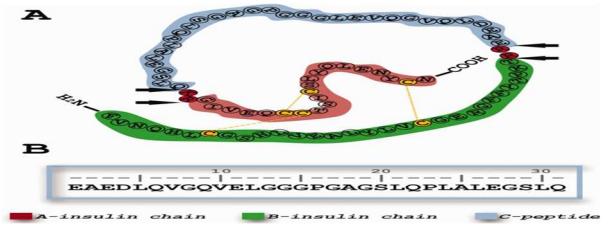

Figure 1.0 Structure of proinsulin. It consist of Insulin A chain (red) and B chain (green) and the connecting peptide. C-peptide (blue) ensures alignment between the A and B chains.

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 cortex C18 column. Analytes and internal standards were detected using the Waters Xevo TQXS 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.

Source (ES+)	Settings
Capillary (kV) 3.32	3.2
Cone (V) 130.90	45
Source Offset (V)	30
Source Temperature (°C)	150
Desolvation Temperature (°C)	600
Cone Gas Flow (L/Hr)	150
Desolvation Gas Flow (L/Hr)	1000
Collision Gas Flow (mL/Min)	0.16
Nebuliser Gas Flow (Bar)	6.7
LM 1 Resolution	1.9
HM 1 Resolution	15.2
Ion Energy 1	0.6
MS Mode Collision Energy	48
LM 2 Resolution	2.9
HM 2 Resolution	15.2
Ion Energy 2	1.2

Table 1.0 MS conditions

Gradient	Time (min)	Flow rate (mL/min)	% Mobile phase A	%Mobile Phase B
1	initial	0.250	85	15
2	4.0	0.250	85	15
3	4.3	0.250	75	25
4	5.0	0.250	65	35
5	5.3	0.250	55	45
6	5.5	0.250	35	65
7	5.7	0.250	15	85
8	8.0	0.250	85	15

Table 2.0 LC conditions

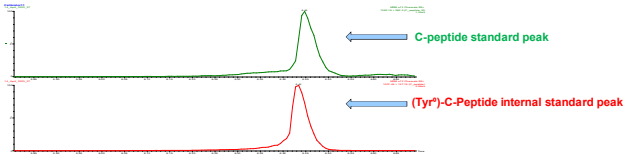

Figure 2.0 Typical chromatogram for C-peptide showing quantitative m/z transitions . a. standard) transition 1007.46>147.19 with retention time (RT) 6.43 min b. (Tyr⁰)-C-Peptide internal standard) transition 1062 >260,20 with retention time (RT)6.45 min

RESULTS

Serum/Plasma - Method Comparison

- The comparison with Mercodia Elisa for serum/plasma shows a positive linear relationship and dispersion of results with an R^2 value of 1.0 and a slope of 0.9378 being achieved.

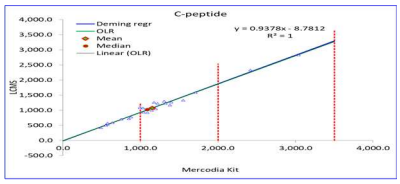

Figure 3.0 Patient sample bias plot showing the linear regression graph between the LCMS method and Mercodia ELISA kit.

Serum/Plasma - Measurement Trueness

- Data from 14 UK NEQAS distributions showed no significant difference from the ALTM despite a slight negative bias.
- The Z-scores were all within +/-2 which was acceptable.

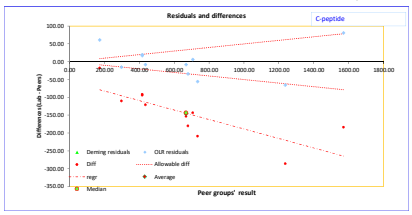

Figure 4.0 Residuals and Difference plots for UK NEQAS Guilford peptide hormone scheme samples

Serum/Plasma- Calibration Curve

- Calibration was prepared in bovine serum for serum and calf whole blood for DBS.
- Data from calibration curve for serum/plasma shows that the percentage difference between the expected and observed concentrations were within 10%.
- The calibration curve R^2 was 0.9999 which was acceptable.

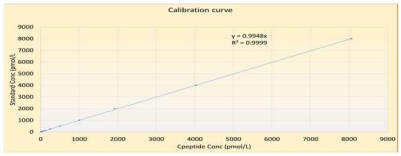

Figure 5.0 Representation of typical Calibration curve showing the corelation coefficient and slope

Serum/Plasma - Linearity

- Linearity of the standard curve was demonstrated by the mean correlation coefficient values for 5 curves from separate runs, The mean R^2 values for C-peptide was greater than 0.99.
- The linearity using a high concentration patient sample demonstrated R^2 value of 0.9994

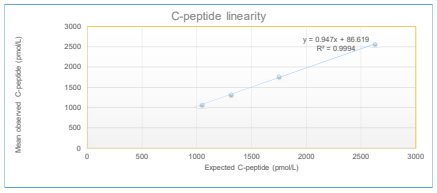

Figure 6.0. Representation of linearity using high concentration patient sample

Serum/Plasma - Inter and Intra assay precision

- To assess the inter-assay precision of the serum/plasma method, a minimum of 5 replicates were tested on different days using both spiked matrix and patient pools over various concentrations of the analyte
- Intra-assay precision was assessed by running 10 replicates of inhouse prepared IQC material within one batch, and over a range of concentrations.
- The CVS was within 10% for all analytes at all concentrations which demonstrates good reproducibility within the assay.

Serum/Plasma - Analytical Sensitivity

- Calibrator 1 (concentration 3.91 pmol/L) achieved a CV of 17.80% which was <20%, this was considered acceptable as the LLOQ. The percentage accuracy against the nominal concentration was <±20%.
- The LOB and LOD was 0.0474pmol/L and 0.08295pmol/L respectively. LOQ was 0.227pmol/L

Serum/Plasma - Matrix effects

- The % matrix effects was <15% apart from the high C-peptide standard spike which was 22% but corrected for by the internal standard.
- Visual inspection of chromatograms shows no areas of ion suppression or enhancement in the region of the retention time of C-peptide and the Internal standard

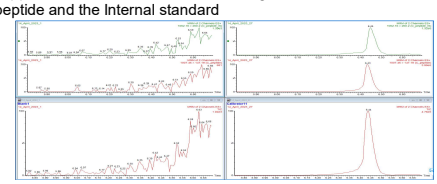

Figure 7.0 Qualitative matrix effect chromatograms

CONCLUSIONS AND FUTURE WORK

Conclusion

In conclusion, an LCMS method has been developed and validated for the determination of C-peptide in serum/plasma and verified for Dry 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 interfering matrix components. Mean percentage carryover was <1% and percentage 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 repertoire at Guildford peptide 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 whilst patients are symptomatic 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

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