**Introduction**

During insulin biosynthesis the proinsulin molecule is cleaved into insulin and C-peptide, both of which are released into the circulation in equimolar amounts. The half-life of C-peptide is approximately 2-5 times higher than that of insulin, making C-peptide a commonly used biomarker of insulin production. Determination of serum C-peptide levels has various clinical applications, including distinguishing type 1 and type 2 diabetes and diagnosing insulin-induced hypoglycemia. In addition, plasma C-peptide levels, 400 ng/mL per centimeter squared, are needed to determine the endogenous insulin production and beta-cell function even in the presence of exogenous administered insulin or anti-insulin antibodies.

Biomarker assays have become a crucial part of clinical studies, emphasizing the need for simple, robust and reliable biochemical methods. Here, a commercially available ELISA kit with recombinant material was used to quantify C-peptide levels in human serum samples.

**Overview Of Method Development**

50 µL of serum, together with BSA- and HRP-conjugated capture and detection antibodies, respectively, are simultaneously added to the wells. The antibody-antigen complex is immobilized to the wells of a microtiter plate pre-coated with Streptavidin. After 2h incubation the wells are washed, and 3,3',5,5'-Tetramethylbenzidine (TMB), a substrate for horse radish peroxidase (HRP), is added. The reaction is stopped by acidification and the absorbance is measured spectrophotometrically at 450 nm. The color intensity is proportional to the C-peptide concentration in the sample. The assay procedure is illustrated in Figure 1.

**Figure 1: Overview of C-peptide ELISA**

**Figure 5:** The effect of plate shaking to the OD (450 nm) response

**Figure 6:** Precision and Accuracy of QCs

**Figure 7:** Parallelism

**Figure 8:** LLOQ 0.15 ng/mL (for concentrations < 1 ng/mL) of C-peptide (Figure 8). Then spiked with either 0.6 ng/mL (for concentrations >1 ng/mL) or 0.3 ng/mL (Figure 9).

**Figure 9:** C-peptide stability in human serum was studied by performing 6 freeze-thaw cycles. A total variation of just 6.9% was calculated.

**Precision And Accuracy**

The between and within run precision as well as accuracy was determined in 2 replicate runs for 3 different quality control (QC) levels (5 duplicates / run), (Figure 6) and for full calibration curves (1 duplicate / run) (Figure 7).

**Selectivity**

To determine possible matrix effect the assay selectivity was tested by first determining the endogenous C-peptide levels from 10 individual samples. Depending on the concentration these individual samples were then spiked with either 0.5 ng/mL, for concentrations < 1 ng/mL or 0.2 ng/mL, for concentrations > 1 ng/mL (Figure 9).

**Analyte Stability**

C-peptide stability in human serum was studied by performing 6 freeze-thaw cycles or keeping the samples at baseline temperature for 3 hours before proceeding with the assay. Stability was demonstrated in all conditions (Figure 10).

**Parallelism**

A hallmark of biomarker assessment is parallelism, a condition in which dilution of a best sample doesn’t result in biased measurements of the analyte concentration. Parallelism was determined by serially diluting 6 individual serum samples containing high (2-0 ng/mL) and low (1 pg/mL) levels of endogenous C-peptide (Figure 10a and 10b). The mean recovery of C-peptide was 102.4%.

**Assay Automation**

To enable high-throughput measurement of serum samples and minimize batch-to-batch errors due to manual pipetting errors the assay was performed using a liquid-handling robot (Tecan Genios FSP 200, Tecan/ThermoFischer) with Tecan Gemini 4.2 Liquid-handling software. The comparison between manual and robotic method is shown in Figure 11.

**Discussion**

In the present work, a novel ELISA method was developed to determine C-peptide levels in human serum. 2 a- and c-chloro-esterase sensitive serum samples were used for the preparation of STDs, and sufficient plate shaking during incubations was shown to be crucial for the assay performance. The robustness of the assay was demonstrated by successfully detecting 6 individual serum samples containing high (2-0 ng/mL) and low (1 pg/mL) levels of endogenous C-peptide.

**Conclusion**

The simplicity, reliability and robustness of the C-peptide assay described here, together with the suitability for method automation, make it an attractive and useful tool for clinical laboratories.