Development of a Highly Sensitive Assay for the Detection of the Biomarker C-Peptide in Human Serum

M. Hyvönen, M. Gröschl, P. Brennecke, P. Struwe.
Celerion Switzerland AG, 8320 Fehraltorf, Switzerland

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Introduction

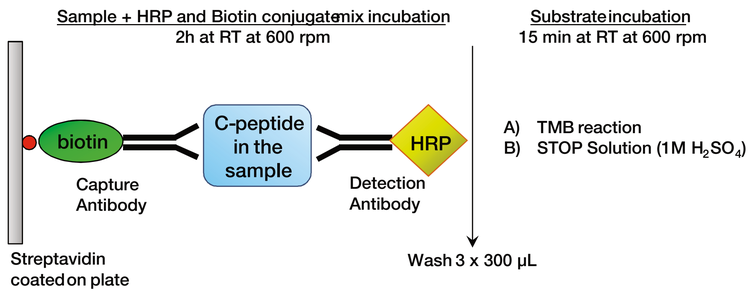
During insulin biosynthesis the proinsulin molecule is cleaved into insulin and C-peptide, both of which are released into the circulation in equimolar amounts. The half-life of C-peptide is approximately 2-5 times higher than that of insulin, making C-peptide a commonly used biomarker of insulin production. Determination of serum C-peptide levels has various clinical applications, including distinguishing type 1 and type 2 diabetes and diagnosing insulin-induced hypoglycaemia. In addition, C-peptide determination allows accurate determination of endogenous insulin production and beta-cell function even in the presence of exogenously administered insulin or anti-insulin antibodies.

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

Overview Of Method Development

50 µL of sample, together with Biotin- 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



The assay specifications are listed in Figure 2 and key reagents used for method development in Figure 3. For the preparation of quality controls (QCs) it was important to choose normal human serum from individuals or pool that contains low endogenous level of C-peptide (here ≤ 0.45 ng/mL).

Figure 2: Assay specifications

Assay Specifications	Description
Analyte	C-peptide
Species	Human
Matrix	Serum
Minimum required dilution (MRD)	1
Analytical range	0.15 – 5 ng/mL
Regression model	4PL
Weighting factor	$1/y^2$

Quality Control (QC)	[ng/mL]
LLOQ	0.15
LQC	0.45
MQC	2
HQC	3.5
ULOQ	5

Figure 3: Key reagents

Reagent ID	Manufacturer, Catalog #	Purpose
C-peptide ELISA kit	Abcam, ab178641	Kit containing streptavidin-coated microtiter plate + capture and detection antibodies
Human C-peptide	NIBSC, 13/146	Reference item
2 x charcoal-stripped human serum	Bioreclamation IVT, HMSRM-STRPD	Matrix for calibration standard preparation
Normal human serum (individual or pool)	Bioreclamation IVT	Matrix for QC preparation Note: use only serum containing ≤ 0.45 ng/mL of endogenous C-peptide

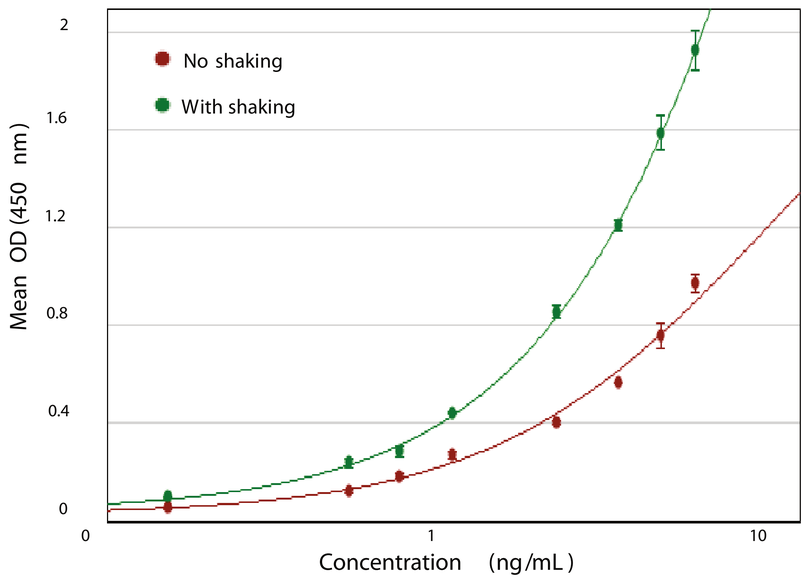
For the preparation of 8 calibrator standards (STDs) an alternative matrix was needed, since normal human serum contains endogenously approx. 0.2 – 2 ng/mL of C-peptide. 2 x charcoal-stripped serum was shown to closely resemble normal human serum after spiking with the analyte, whereas different surrogate matrices gave too low responses and were not suitable for this assay (Figure 4).

Figure 4: Comparison of different matrices for STD preparation

	Nominal [ng/mL]	Mean OD (540nm)	Std. Dev.	CV%	Mean result	%Bias
Normal human serum	1.52	0.261	0.045	2.968	1.525	0.351
	1.62	0.289	0.075	4.478	1.668	2.951
	2.52	0.554	0.067	2.287	2.992	15.935
	11.52	3.108	0.319	2.725	11.718	1.719
2 x charcoal stripped serum	0	0.029	0.003	9.266	range	range
	0.1	0.038	0.002	4.041	0.111	10.934
	1	0.166	0.010	5.763	0.999	-0.089
	10	2.491	2.491	7.734	9.998	-0.025
Sigmatrix (D4197, Sigma-Aldrich)	0	range	range	range	range	range
	0.1	range	range	range	range	range
	1	0.063	0.061	18.909	0.325	-67.511
	10	1.321	0.252	4.202	5.986	-40.139
0.1% BSA in 1 x DPBS	0	range	range	range	range	range
	0.1	range	range	range	range	range
	1	0.053	0.005	2.258	0.239	-76.123
	10	0.875	0.030	0.705	4.275	-57.254

In order to obtain the best signal to noise-ratio, and therefore, the best sensitivity, plate shaking at approx. 600 rounds per minute (rpm) was shown to be crucial (Figure 5). Shaking also enables a higher analytical range.

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



Precision And Accuracy

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

Figure 6: Precision and Accuracy of QCs

Nominal [ng/mL]	QC LLOQ 0.15	QC Low 0.45	QC Med 2.0	QC High 3.5	QC ULOQ 5.0
%Bias	-3.9	11.1	12.5	5.0	2.1
Between Run Precision (%CV)	0.0	0.0	4.0	7.1	7.1
Within Run Precision (%CV)	7.6	3.9	2.1	1.9	2.1
Total Variation (%CV)	6.5	3.3	4.5	7.4	7.4

Figure 7: Precision and Accuracy of calibration curve

Nominal [ng/mL]	STD1 0.15	STD2 0.5	STD3 0.7	STD4 1	STD5 2	STD6 3	STD7 4	STD8 5
Mean	0.16	0.50	0.70	0.94	2.02	3.23	4.19	4.73
SD	0.01	0.02	0.03	0.04	0.02	0.23	0.05	0.11
%CV	4.32	4.79	4.50	3.81	1.15	7.18	1.20	2.26
%Bias	105.1	100.3	99.4	93.7	100.9	107.69	104.7	94.7

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 0.6 ng/mL (for concentrations >1 ng/mL) or 0.3 ng/mL (for concentrations < 1 ng/mL) of C-peptide (Figure 8).

Figure 8: Assay selectivity

Individual	Blank [ng/mL]	Spike [ng/mL]	Response [ng/mL]	Nominal %
1	1.159	0.6	1.832	104.2
2	0.96	0.6	1.396	89.5
3	0.768	0.3	1.065	99.7
4	0.549	0.3	0.838	98.7
5	0.358	0.3	0.653	95.3
6	1.034	0.6	1.763	107.9
7	0.194	0.3	0.524	106.1
8	0.614	0.3	0.967	105.8
9	0.314	0.3	0.702	114.3
10	1.365	0.6	1.873	95.3
mean	0.732			101.7
SD	0.391			7.3
CV [%]	53.5			7.2

Analyte Stability

C-peptide stability in human serum was studied by performing 6 freeze-thaw cycles or keeping the samples at benchtop temperature for 3 hours before proceeding with the assay. Stability was demonstrated in both conditions (Figure 9).

Figure 9: C-peptide stability in human serum

Nominal [ng/mL]	QC Low 0.450 [ng/mL]		QC High 3.500 [ng/mL]	
	mean recovery [%]	%Bias < 20%	mean recovery [%]	%Bias < 20%
Benchtop 3 hours	102.1	3/3 acceptable	112.3	3/3 acceptable
Freeze / Thaw 6 cycles	103.0	3/3 acceptable	98.4	3/3 acceptable

Parallelism

A hallmark of biomarker assessment is parallelism, a condition in which dilution of a test sample doesn't result in biased measurements of the analyte concentration. Parallelism was successfully demonstrated by serially diluting 6 individual serum samples containing high (>2.5 ng/mL) endogenous levels of C-peptide (Figure 10a and 10b). The mean recovery of C-peptide was 102.4%.

Figure 10a: Parallelism

Individual	Dilution Factor	Back-calculated conc. [ng/mL]	conc. [ng/mL]	Recovery %	%CV	%Bias
1	undiluted	4.111	4.111	100.00	1.85	
	2	2.155	4.31	104.84	1.54	4.84
	4	1.13	4.52	109.95	2.65	9.95
	8	0.496	3.968	96.52	0.33	-3.48
		Mean	4.23			
		SD	0.24			
		%CV	5.69			
2	undiluted	3.978	3.978	100.00	0.60	
	2	2.242	4.484	112.72	0.00	12.72
	4	0.96	3.84	96.53	0.90	-3.47
	8	0.394	3.152	79.24	0.50	-20.76
		Mean	3.86			
		SD	0.55			
		%CV	14.22			
3	undiluted	3.894	3.894	100.00	0.50	
	2	2.125	4.25	109.14	1.10	9.14
	4	1.106	4.424	113.61	0.20	13.61
	8	0.537	4.296	110.32	0.00	10.32
		Mean	4.22			
		SD	0.23			
		%CV	5.38			

Figure 10b: Parallelism

Individual	Dilution Factor	Back-calculated conc. [ng/mL]	conc. [ng/mL]	Recovery %	%CV	%Bias
4	undiluted	3.782	3.782	100.00	0.50	
	2	1.895	3.79	100.21	1.10	0.21
	4	1.019	4.076	107.77	0.20	7.77
	8	0.489	3.912	103.44	0.00	3.44
		Mean	3.89			
		SD	0.14			
		%CV	3.54			
5	undiluted	3.114	3.114	100.00	0.50	
	2	1.531	3.062	98.33	1.10	-1.67
	4	0.723	2.892	92.87	0.20	-7.13
	8	0.345	2.76	88.63	0.00	-11.37
		Mean	2.96			
		SD	0.16			
		%CV	5.48			
6	undiluted	2.576	2.576	100.00	0.50	
	2	1.354	2.708	105.12	1.10	5.12
	4	0.72	2.88	111.80	0.20	11.80
	8	0.377	3.016	117.08	0.00	17.08
		Mean	2.80			
		SD	0.19			
		%CV	6.90			

Assay Automatization

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

Figure 11: Comparison of manual and robotic methods

	Sample	Nominal [ng/mL]	Mean Result	Std. Dev.	CV%	%Bias
Manual Method	LLOQ	0.15	0.162	0.003	1.685	8.277
	LQC	0.45	0.482	0.003	0.657	7.102
	MQC	2	2.171	0.065	2.991	8.54
	HQC	3.5	3.308	0.108	3.258	-5.493
	ULOQ	5	4.828	0.215	4.462	-3.43
Robotic Method	LLOQ	0.15	0.124	0.005	3.789	-17.642
	LQC	0.45	0.463	0.036	7.863	2.806
	MQC	2	1.922	0.002	0.110	-3.895
	HQC	3.5	3.079	0.064	2.078	-12.018
	ULOQ	5	4.615	0.229	4.964	-7.702

Discussion

In the present work, a sandwich ELISA method was developed to determine C-peptide levels in human serum. 2 x charcoal-stripped serum was suitable for the preparation of STDs, and sufficient plate shaking during incubations was shown to be crucial for the assay sensitivity. Importantly, the parallelism of endogenous C-peptide in human serum was successfully demonstrated. Therefore, the assay is highly suitable for the detection of this important biomarker.

Conclusion

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

Poster presentation at EBF 2017