Research Article

Development and validation of a novel LC-MS/MS assay for C-peptide in human serum

Benjamin Y. Owusu,1, Hannah Pflaus,2, Russell Garner,1, North Foulon,1, Thomas J. Lahaa, Andrew N. Hoofnagle1,⇑

1 Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, United States
2 Department of Medicine, University of Washington, Seattle, WA, United States

A R T I C L E   I N F O
Article history:
Available online 22 December 2020

Keywords:
C-peptide
Serum
Validation
Liquid chromatography-tandem mass spectrometry
LC-MS/MS

A B S T R A C T

Introduction: C-peptide is used as a marker of endogenous insulin secretion in the assessment of residual β-cell function in diabetes and in the diagnostic workup of hypoglycemia. Previously developed LC-MS/MS methods to quantify serum concentrations of C-peptide have monitored intact peptide, which ionizes poorly. As a result, methods have leveraged immunoaffinity enrichment or two-dimensional chromatography. In this study, we aimed to use proteolysis during sample preparation to enhance the sensitivity of traditional LC-MS/MS.

Methods: Due to the absence of arginine and lysine residues in C-peptide, we utilized Glu-C as the proteolytic enzyme in the method. After protein precipitation using acetonitrile and solid phase extraction with mixed anion exchange, lower molecular weight polypeptides were reduced, alkylated, and proteolyzed. The two amino-terminal peptide fragments, EAEDLQVGQVE and LGGGPGAGSLQPLALE, were monitored using multiple reaction monitoring in positive ion mode (Acquity ULPC-Xevo TQ-S, Waters). The former peptide was used for quantification and the latter for quality assurance.

Results: Glu-C was determined to be a reliable proteolytic enzyme with monotonic digestion kinetics. The assay was linear between 0.1 and 15 ng/mL and had a lower limit of quantification of 0.06 ng/mL. Total imprecision was 7.7 %CV and long-term imprecision at 0.16 ng/mL was 10.0%. Spike-recovery experiments demonstrated a mean recovery of 98.2 % (± 9.1 %) and the method compared favorably with a commercially available immunoassay and a reference measurement procedure.

Conclusion: Protein precipitation with solid phase extraction and proteolysis with Glu-C is a robust sample preparation method for quantification of C-peptide in human serum by LC-MS/MS.

1. Introduction

C-peptide is a relatively short polypeptide of 31 amino acids that is derived from proinsulin, a prohormone synthesized in the β-cells of the pancreatic islets. After proinsulin is processed by prohormone convertases and carboxypeptidase E in the Golgi, insulin and C-peptide are stored in secretory granules until the cells are stimulated to secrete insulin by increased blood glucose concentra-

1 Present address: Seattle Children’s Hospital, Seattle, WA 98105, United States.
2 Corresponding author at: Campus Box 357110, 1959 NE Pacific St. NW120, University of Washington, Seattle, WA 98195-7110, United States.
E-mail address: ahoof@uw.edu (A.N. Hoofnagle).

Abbreviations: LC-MS/MS, liquid chromatography-tandem mass spectrometry; LLOQ, lower limit of quantification; %CV, coefficient of variation expressed as a percent.

Peer review under responsibility of MSACL

© 2020 THE AUTHORS. Publishing services by ELSEVIER B.V. on behalf of MSACL. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
concentration of C-peptide in healthy individuals (0.3–3 nM, 0.9–9 ng/mL) is approximately 5-times higher than insulin [4]. Due to the extent of renal metabolism, C-peptide concentrations are higher in patients with later stages of kidney disease [2].

While C-peptide may have some therapeutic potential in the future, its plasma concentration is currently used clinically to help determine the pathophysiological processes leading to hyperglycemia and hypoglycemia [2–4]. Pharmaceutical preparations of insulin and its analogs do not contain C-peptide and, as a result, C-peptide plays an important role in detecting endogenous insulin secretion [8]. This can be useful in demonstrating elevated insulin secretion in hyperglycemic patients with insulin resistance and type 2 diabetes or in identifying inappropriate insulin secretion in hypoglycemic patients with insulin secreting tumors. In contrast, a lack of C-peptide in circulation is useful in detecting β-cell loss in hyperglycemic patients and in identifying patients who have administered exogenous insulin in the case of unexplained hypoglycemia. In patients with type 1 diabetes, C-peptide is used in research settings as a measure of residual β-cell function (often after stimulation with oral glucose) and less commonly in clinical settings for prognosis.

As an analyte, C-peptide is most commonly detected and quantified using immunoassays. While modern immunoassays are sensitive, concerns regarding specificity, e.g., cross-reactivity with proinsulin, and the poor inter-platform concordance have been raised [9,10]. In many ways, mass spectrometric assays have the potential to overcome the limitations of immunoassays [11–15]. In addition, detailed standard operating procedures will facilitate the transfer of mass spectrometric technology between laboratories [16]. Previous mass spectrometric assays for the quantification of serum C-peptide analyzed intact peptide, which has low ionization efficiency, thus limiting its analytical sensitivity on many instruments. This limited ionization efficiency has been overcome by the use of multiple dimensions of liquid chromatographic separation [17,18] or with immunoaffinity enrichment [19,20]. In this study, we aimed to develop and validate a new assay for C-peptide using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with standard chromatography and no immunoaffinity enrichment.

2. Materials and methods

A detailed standard operating procedure is presented in the Supplemental Material. A brief description of the method is provided in Sections 2.2 and 2.3.

2.1. Description of samples

De-identified leftover clinical samples were obtained from the Chemistry Laboratory at the University of Washington Medical Center. The use of de-identified leftover samples and samples drawn for quality improvement has been determined to qualify as non-human subjects-research by the Human Subjects Division of the University of Washington.

2.2. Sample preparation

Briefly, samples (200 µL) and internal standard (C-peptide labeled with two heavy isotope-labeled amino acids) were precipitated with acetonitrile in a filter plate (Pall) and filtered using centrifugation. We noticed early on in method development that C-peptide was being lost due to adsorption in standard buffers, including 5–10% acetonitrile with and without formic acid. However, after testing different buffer conditions, we found that the analyte was stable in 0.001% Zwittergent 3–16 detergent. As a result, this storage solution was adopted for C-peptide. The filtrate was dried using centrifugal evaporation at room temperature. Samples were reconstituted, acidified, and enriched using mixed anion exchange solid-phase extraction with a μ-elution plate (Waters). The eluate was dried at 40 °C using evaporation (TurboVap, Biotage), reconstituted in a buffer containing dithiothreitol, and reduced at 60 °C. After cooling and alkylation with iodoacetamide, samples were digested with endoproteinase Glu-C (Sigma) at 37 °C at pH 8. The specificity and reliability of digestion was determined to be acceptable in the pH range of 7.5–9.5 (Supplemental Figure 1). The reaction was stopped with formic acid.

2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

A portion of the digests were injected onto the LC-MS/MS instrument and peptides were resolved using reverse phase chromatography at normal flow rates (Waters Acquity). Resolved peptides were analyzed with a Waters Xevo TQ-S tandem mass spectrometer. The two amino-terminal peptides and their internal standards were monitored, EAEDLVQGVQVE and LGGPGAGSLQPLALE (underlined leucines were labeled in the internal standard C-peptide). The peak area ratio of peptide EAEDLVQGVQVE was used for quantification and that of peptide LGGPGAGSLQPLALE was used for quality assurance (as a confirmatory ion). The data were analyzed with MassLynx and TargetLynx XS. Example product ion spectra and chromatograms are included in Supplemental Figures 2 and 3, respectively. The assay was calibrated using a five-point calibration curve, which was made from a combination of pooled human serum, synthetic human C-peptide, and horse serum (negative for the peptides of interest).

2.4. Method validation

The analytical method validation experiments included evaluation of linearity, imprecision, lower limit of quantification, carryover, spike-recovery, interference studies, and method comparison. Each batch of samples was prepared in parallel with a 5-point standard curve and negative (equine serum), low (0.15 ng/mL), and high (5.0 ng/mL) quality control materials. System suitability was assessed each day by using a pooled processed sample with a low concentration of C-peptide. All statistical analyses were performed in Excel 2016 and R (version 4.0.0) [21].

2.4.1. Linearity

The linearity of the assay was assessed using an 11-point dilution series ranging from 0.1 to 15 ng/mL. The sample with a high concentration of C-peptide (14.3 ng/mL) was prepared by spiking a pool of leftover clinical samples with synthetic C-peptide certified reference material (CRM, NMJ CRM 6901-b, Wako Chemicals USA). The sample with a low concentration of C-peptide (0.2 ng/mL) was prepared by dilution of a pool of leftover clinical samples with equine serum. The 11-point dilution series was constructed volumetrically by mixing these two samples together in appropriate ratios. Samples were run in triplicate on each of 3 days. The Pearson correlation coefficient (r²) and bias for the middle nine samples were determined using Excel.

2.4.2. Imprecision

We evaluated within-batch imprecision by analyzing 20 replicates of pooled leftover clinical samples with low (0.1 ng/mL), medium (5.9 ng/mL) and high (11.7 ng/mL) concentrations of C-peptide in a single batch. To estimate total variability, we performed a 5 × 5 study by analyzing 5 replicates of a sample with 0.3 ng/mL C-peptide on each day for 5 days. To estimate between-batch imprecision, we analyzed 20 replicates of a sample...
with 0.2 ng/mL over 4 months. Data for imprecision are presented as the coefficient of variation expressed as a percent (%CV).

2.4.3. Lower Limit of Quantification (LLOQ)

The LLOQ was estimated by analyzing 8 replicates of 5 different samples that had low concentrations of C-peptide (0.21, 0.14, 0.07, 0.04, and 0.02 ng/mL, made by mixing leftover clinical samples) on each day for 5 days. The concentration at which the imprecision was interpolated to be 20 %CV (using regression with a power function in Excel) was estimated to be the LLOQ.

2.4.4. Carryover

The extent of carryover was evaluated by alternating injections of digests of pooled leftover clinical samples with high and low concentrations of C-peptide sequentially in triplicate. Carryover was calculated as the ratio of the difference between the mean peak area of the low samples that immediately followed the high samples and the mean peak area of the low samples that immediately preceded the high samples, divided by mean peak area of the high sample that was injected second in each series of triplicate injections (expressed as a percentage).

2.4.5. Spike-recovery and Interference

For spike-recovery studies, leftover clinical samples (n = 25) were spiked with 5 ng/mL C-peptide CRM or buffer (control). The effects of hemolysis, lipemia, bilirubin, kidney disease, and total protein were also evaluated by spiking potentially problematic samples in a similar manner. Spiked samples (with peptide or buffer) were extracted in duplicate.

2.4.6. Sample type and stability

To compare results from different phlebotomy tubes, blood was drawn into lime green-top serum separator tubes (lithium-heparin), gold-top serum separator tubes, red-top serum tubes, and lavender-top plasma tubes (EDTA anticoagulated) during the same phlebotomy (all blood was collected into non-expired BD Vacutainers). Blood from three healthy individuals was available for this study. Short-term storage stability of C-peptide was evaluated at room temperature (24 hr), 4 °C (48 hr), and -20 °C (1 week). Samples were also subjected to two freeze-thaw cycles.

2.4.7. Method Comparison

Leftover clinical samples that were previously tested using the Immulite 1000 immunoassay (Siemens, N = 38) or the reference measurement procedure at the University of Missouri [17,18] (N = 40) were used in a method comparison. For comparison with immunoassay, Deming regression was performed (with the deming function in the deming package in R, assuming equal variance for the immunoassay and the new assay). For comparison with the reference method, standard linear regression in R was used for method comparison (setting the reference method as the gold standard). The comparison with the immunoassay was performed over two days by the same operator and the comparison with the reference measurement procedure spanned two operators and 6 months.

2.4.8. Quality assurance

Acceptance criteria were established for the retention time of EAE and LGG peptides (3.04–3.42 min and 3.84–4.36 min, respectively), the correlation coefficient of the calibration curve (≥0.98), the observed concentration of the low (0.15 ng/mL) and high (2.75 ng/mL) concentrations of C-peptide, the ratio of the two peptides (EAE/LGG, 0.4–0.6), and the transition ion ratios for the EAE peptide (785.37/686.30, 1.5–2.1; 785.37/659.34, 2.6–3.2; 785.37/531.28, 2.0–2.8). Samples fail more than one of the quality assurance parameters –2% of the time, at which point the samples are reviewed and may be re-injected or repeated.

3. Results

There are no trypsin sites in C-peptide, which prevented us from taking advantage of this commonly-used proteolytic enzyme. Instead, we used Glu-C, a protease that cleaves peptides on the carboxyl-terminal side of glutamate and aspartate residues. This digestion was performed after solid phase extraction with a mixed anion exchange resin, reduction with diithiothreitol, and alkylation with iodoacetamide. While C-peptide does not contain cysteines, one of our goals is to multiplex the measurement of C-peptide with other analytes that contain cysteines, and as a result, we included reduction and alkylation in the method. Intact isotopically labelled C-peptide was used as an internal standard and was spiked in before extraction and digestion. The two amino-terminal peptides were of interest, with EAEDLOVGVQE monitored for quantification and LGGPAGASLQPLALE monitored for quality assurance. The proteolytic release of the two peptides reached a plateau within an hour under these digestion conditions (Figure 1, Supplemental Figure 4). There was minimal ion suppression observed with this method (Supplemental Figure 5).

3.1. Linearity

The linearity of the assay was determined using an 11-point dilution series prepared by proportionally mixing a pool of leftover clinical samples with a high C-peptide concentration with a pool of C-peptide-deficient serum. The assay was linear between 0.1 and 15 ng/mL, with a correlation coefficient (Pearson $r^2$) of 0.997, and observed biases of -10% to 7% for each mixture (Figure 2).

3.2. Imprecision

Within-batch imprecision of the assay was determined to be 11.2 %CV, 3.7 %CV and 3.8 %CV at low (0.1 ng/mL), medium (5.9 ng/mL) and high (11.7 ng/mL) concentrations of C-peptide (N=20), respectively. Total variability was estimated using a 5x5 imprecision study, which analyzed 5 replicates of a serum pool (0.3 ng/mL) each day for 5 days (Supplemental Table 1). From this experiment, the within-batch and between-batch imprecision were estimated to be 5.3 %CV and 5.5 %CV, respectively, and the total imprecision was estimated to be 7.7 %CV. Between-batch imprecision was determined to be 11.8 %CV at 0.16 ng/mL (N=20 days over four months).

3.3. Lower limit of quantification

The LLOQ of the assay was estimated by analyzing 8 replicates of 5 different samples with low concentrations of C-peptide (0.02, 0.04, 0.07, 0.14 and 0.21 ng/mL) on each day for 5 days (Supplemental Figure 6). From this experiment, the LLOQ was estimated to be 0.06 ng/mL. There was one outlier replicate in this analysis, which when removed did not substantially change the estimated LLOQ (0.05 ng/mL).

3.4. Instrument carryover

Carryover was evaluated by alternating triplicate injections of digests with high (50 ng/mL) and low (0.5 ng/mL) concentrations of proteolytic peptides derived from C-peptide (Supplemental Table 2). The observed carryover, 0.06%, was not significant enough to cause concerns for analysis of human samples.
3.5. Spike-recovery and potential interference

We evaluated the recovery of spiked C-peptide in leftover clinical samples that did not have detectable amounts of known potential interferences and those that did. The mean recovery in samples that did not appear to have interferences was 98.2% (SD = 9.1%, range 73–113.5, Figure 3). There was one outlier replicate in this experiment. When removed, the mean recovery did not substantially change (98.8%, Supplemental Figure 7). No systematic concentration-dependent interference was observed from leftover clinical samples with hemoglobin up to 0.84 g/dL, triglycerides up to 1850 mg/dL, total protein up to 11.4 g/dL, bilirubin up to 33 mg/dL, or creatinine up to 10.8 mg/dL (Supplemental Figure 8).

3.6. Tube-type and stability studies

To evaluate the impact of different matrices and preanalytical handling on the quantification of C-peptide, blood was collected from three volunteers in four different collection tube types and incubated at different temperatures (Supplemental Table 3). The concentration of C-peptide that was observed for freshly analyzed serum from a red-top tube served as the reference for each volunteer. There was no substantial bias observed for EDTA-anticoagulated plasma, lithium-heparin anticoagulated plasma collected in a gel separator tube, or serum collected in a gel separator tube (bias ranged from –7.3% to 4.4%). There was also little impact when the tubes were incubated for 24 hr at room temperature, 48 hr at 4°C, or one week at –20°C (bias ranged from –7.2% to 7.2%), or when the samples were put through a second freeze-thaw cycle (bias ranged from –0.9% to 7.2%).

3.7. Method comparison

Comparison between the new method and a commercially available immunoassay (Siemens Immulite 1000) revealed a favorable correlation with Pearson $r^2 = 0.972$ and a Deming regression equation of New Method = 0.986 * Immunoassay + 0.255 (Figure 4A). The mean relative difference across all samples was 0.19 ng/mL or 3.5% (Supplemental Figure 9). Comparison between the new method and the reference measurement procedure at the University of Missouri showed more scatter, with Pearson $r^2 = 0.934$ and linear regression equation of New Method = 0.960 * Reference Method + 0.227 (Figure 4B). The mean relative difference across all samples was 0.04 ng/mL or 2.5% (Supplemental Figure 9).

4. Discussion

We have developed a new method for the quantification of C-peptide in human serum. The method avoids the use of immunoaffinity enrichment and multidimensional chromatography and instead uses proteolysis following protein precipitation and a single solid phase extraction step. The method is sensitive,
linear, precise, and has good agreement with comparator methods. It provides an alternative to immunoassays, has a throughput acceptable for use in clinical research or clinical care, and can form the basis for extending standardization efforts and further assay development. We have launched the assay for use in clinical care and it has performed robustly (long-term imprecision is 12.5 %CV at 0.15 ng/mL and 8.4% at 2.75 ng/mL over 39 production runs). The method is performed in a 96-well plate format and requires approximately 2.5 h of dedicated scientist time over two days (assembly preparation requires 8 h, including incubation steps). While some steps are manual, much of the sample handling is amenable to automation.

In contrast with previous methods, this assay did not monitor intact C-peptide, but instead quantified a proteolytic fragment of C-peptide. While trypsin is used in many proteomics settings, there are no lysine or arginine residues in C-peptide, which are the target residues for trypsin proteolysis. In contrast, there are many glutamic and aspartic acids in C-peptide, so we utilized Glu-C; and while the specificity of Glu-C is a function of buffer and pH, it turned out to be reproducible with our standard operating procedure.

Previous assays have used selected ion monitoring [18,22], multiple stages of solid phase extraction or liquid chromatography [17,23], or immunoaffinity enrichment to achieve the sensitivity needed for the assay [20,22]. We found that the ionization efficiency of the two Glu-C peptides at the amino-terminus of C-peptide was significantly better than intact C-peptide on our instruments.

Although ionization efficiency was superior for the Glu-C peptides, the most abundant ion observed carried a single positive charge, which is consistent with the lack of basic residues in each peptide. Due to the fact that the precursor ion has a higher m/z than the fragment ion, there could be concern regarding a lack of specificity for the quantitative transitions. Indeed, many proteomics methods take advantage of precursor ions that have a lower m/z than the fragment, which reduces noise. In our sample preparation workflow, we have included protein precipitation and solid phase extraction, which greatly simplify the matrix injected onto the LC-MS/MS. As an additional safeguard, we have established three transition ion ratios, and one peptide ratio, to help identify instances when an interfering ion is present. It is important for new methods to include similar quality assurance parameters, particularly if they monitor singly charged peptides.

While the clinical use of C-peptide is limited, many ongoing clinical research studies have suggested an active biological role for C-peptide in both preventing diabetic complications [2–4,24] and as a biomarker to predict response to therapeutic interventions [25–27]. The poor concordance between immunoassay platforms has delayed the clinical implementation of routine C-peptide monitoring [8–10]. It has been proposed that this problem be addressed by a reference measurement system [28]. Our initial method comparison with the reference measurement procedure in use at the University of Missouri [17] is encouraging and suggests that, if laboratories are able to adopt our detailed standard operating procedure, these centers could play an important role in improving standardization across research and clinical laboratories. To this end, the National Institute for Diabetes, Digestive, and Kidney Diseases has recently formed the Targeted Mass Spectrometry Assays for Diabetes and Obesity Research (TaMaDOR) Working Group, which will attempt to translate this method to other laboratories. It will be interesting to assess measures of reproducibility and accuracy across other centers. Also, as part of this Working Group, the method will be expanded to include other analytes in a multiplexed fashion, with the intent of making mass spectrometry assays more accessible to the clinical research community studying diabetes and obesity.

5. Conflict of Interest Statement

Andrew N. Hoofnagle has served as an expert witness on mass spectrometry for Kilpatrick Townsend & Stockton LLP. This work was supported by NIDDK/NIH grants U01 DK121289 and P30 DK017047. Equipment and research funding has been provided to the University of Washington by Waters, Inc., and is related to the work presented here.

Declaration of Competing Interest

Andrew N. Hoofnagle has served as an expert witness regarding mass spectrometry for Kilpatrick Townsend and Stockton LLP. The remaining authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2020.12.001.

References

[1] H.W. Davidson, C.J. Rhodes, J.C. Hutton, Intraorganelar calcium and pH control proinsulin cleavage in the pancreatic beta cell via two distinct site-specific endopeptidases, Nature 333 (1988) 93–96.
[2] R.C. Marques, M.J. Fontaine, J. Rogers, C-peptide: much more than a byproduct of insulin biosynthesis, Pancreas 29 (2004) 231–238.
[3] G.L. Yosten, G.R. Kolar, The physiology of proinsulin c-peptide: unanswered questions and a proposed model, Physiology (Bethesda) 30 (2015) 327–332.
[4] G.L.C. Yosten, C. Marie-Blikan, P. Luppi, J. Wahren, Physiological effects and therapeutic potential of proinsulin C-peptide, Am. J. Physiol.-Endocrinol. Metabolism 307 (11) (2014) E955–E968, https://doi.org/10.1152/ajpendo.00130.2014.

[5] J.H. Henriksen, B. Tronier, J.B. Bulow, Kinetics of circulating endogenous insulin, C-peptide, and proinsulin in fasting nondiabetic man, Metabolism 36 (1987) 463–468.

[6] E. Melles, H. Jornvall, S. Tryggvason, K.G. Danielsson, K. Ekberg, K. Tryggvason, J. Wahren, T. Bergman, Degradation of proinsulin C-peptide in kidney and placenta extracts by a specific endoprotease activity, Cell Mol. Life Sci. 61 (2004) 2979–2982.

[7] I. Zavaroni, G. Defferrari, R. Lugarri, E. Bonora, G. Caribotti, E. Dall’Aglio, C. Robaudo, A. Gnudi, Renal metabolism of C-peptide in man, J. Clin. Endocrinol. Metab. 65 (1987) 494–498.

[8] A.G. Jones, A.T. Hattersley, The clinical utility of C-peptide measurement in the care of patients with diabetes, Diabet. Med. 30 (2013) 803–817.

[9] R.R. Little, C.L. Rohlfing, A.L. Tennill, R.W. Madsen, K.S. Polonsky, G.L. Myers, C.J. Greenbaum, J.P. Palmer, E. Rogatsky, D.T. Stein, Standardization of C-peptide measurements, Clin. Chem. 54 (2008) 1023–1026.

[10] H.M. Wiedmeyer, K.S. Polonsky, G.L. Myers, R.R. Little, C.J. Greenbaum, D.E. Goldstein, J.P. Palmer, International comparison of C-peptide measurements, Clin. Chem. 53 (2007) 784–787.

[11] H.D. Cox, F. Lopes, G.A. Woldemariam, J.O. Becker, M.C. Parkin, A. Thomas, A.W. Butch, D.A. Cowan, M. Thevis, L.D. Bowers, A.N. Hoofnagle, Interlaboratory agreement of insulin-like growth factor 1 concentrations measured by mass spectrometry, Clin. Chem. 60 (2014) 541–548.

[12] A.N. Hoofnagle, M.H. Wener, The fundamental flaws of immunoassays and potential solutions using tandem mass spectrometry, J. Immunol. Methods 347 (2009) 3–11.

[13] M. Moncrieffe, H.D. Cox, S. Carletta, J.O. Becker, A. Thomas, D. Eichner, B. Ahrens, M. Thevis, L.D. Bowers, D.A. Cowan, A.N. Hoofnagle, Inter-Laboratory agreement of insulin-like growth factor 1 concentrations measured intact by mass spectrometry, Clin. Chem. 66 (2020) 579–586.

[14] B.C. Netzel, R.P. Grant, A.N. Hoofnagle, A.L. Rockwood, C.M. Shuford, S.K. Grebe, First steps toward harmonization of LC-MS/MS thyroglobulin assays, Clin. Chem. 62 (2016) 297–299.

[15] B.C. Netzel, S.K. Grebe, B.G. Carranza Leon, M.R. Castro, P.M. Clark, A.N. Hoofnagle, C.A. Spencer, A.F. Turcu, A. Algeciras-Schimnich, Thyroglobulin (Tg) testing revisited: Tg Assays, TgAb assays, and correlation of results with clinical outcomes, J. Clin. Endocrinol. Metab. 100 (2015) E1074–E1083.

[16] R.P. Grant, A.N. Hoofnagle, From lost in translation to paradise found: enabling protein biomarker method transfer by mass spectrometry, Clin. Chem. 60 (2014) 941–944.

[17] K. Kalyantay, A. Durairaj, D. Shin, C.L. Rohlfing, S. Connolly, R.R. Little, A.V. Stoyanov, Two-step ion-exchange chromatographic purification combined with reversed-phase chromatography to isolate C-peptide for mass spectrometric analysis, J. Sep. Sci. 39 (2016) 676–681.

[18] A.V. Stoyanov, C.L. Rohlfing, S. Connolly, M.L. Roberts, C.L. Nauser, R.R. Little, Use of cation exchange chromatography for human C-peptide isolation and measurement, J. Chromatogr. A 1218 (2011) 9244–9249.

[19] T. Kinum, R. Mizuno, A. Takatsu, Quantification of serum C-peptide by isotope-dilution liquid chromatography–tandem mass spectrometry: Enhanced detection using chemical modification and immunoonaffinity purification, J. Chromatogr. B 953–954 (2014) 138–142, https://doi.org/10.1016/j.jchromb.2014.02.019.

[20] S.W. Taylor, N.J. Clarke, Z. Chen, M.J. McPhaul, A high-throughput mass spectrometry assay to simultaneously measure intact insulin and C-peptide, Clin. Chem. Acta 455 (2016) 202–208.

[21] R Core Team, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, 2020.

[22] A.D. Kippen, F. Cerini, L. Vadas, R. Stocklin, L. Vu, R.E. Offord, K. Rose, Development of an isotope dilution assay for precise determination of insulin, C-peptide, and proinsulin levels in non-diabetic and type II diabetic individuals with comparison to immunonaassay, J. Biol. Chem. 272 (1997) 12513–12522.

[23] E. Rogatsky, B. Balent, G. Goswami, V. Tomuta, H. Jayatillake, G. Cruikshank, L. Vele, D.T. Stein, Sensitive quantitative analysis of C-peptide in human plasma by 2-dimensional liquid chromatography-mass spectrometry isotope-dilution assay, Clin. Chem. 52 (2006) 872–879.

[24] J.P. Richards, E.A. Bowles, W.R. Gordon, M.L. Ellsworth, A.H. Stephenson, R.S. Sprague, Mechanisms of C-peptide-mediated rescue of low O2–induced ATP release from erythrocytes of humans with Type 2 diabetes, Am. J. Physiol.-Regul., Integrative Comparative Physiol. 308 (5) (2015) R411–R418, https://doi.org/10.1152/ajpregu.00420.2014.

[25] M.M. Bogun, B.N. Bundy, R.S. Goland, C.J. Greenbaum, C-peptide levels in subjects followed longitudinally before and after type 1 diabetes diagnosis in TrialNet, Dia Care 43 (8) (2020) 1836–1842, https://doi.org/10.2337/dc19-2288.