Multiplexed quantification of insulin and C-peptide by LC-MS/MS without the use of antibodies

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ABSTRACT

Introduction: The measurement of insulin and C-peptide provides a valuable tool for the clinical evaluation of hypoglycemia. In research, these biomarkers are used together to better understand hyperinsulinemia, hepatic insulin clearance, and beta cell function. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an attractive approach for the analysis of insulin and C-peptide because the platform is specific, can avoid certain limitations of immunoassays, and can be multiplexed. Previously described LC-MS/MS methods for the simultaneous quantification of insulin and C-peptide measure the intact analytes and most have relied on immunoaffinity enrichment. These approaches can be limited in terms of sensitivity and interference from autoantibodies, respectively. We have developed a novel method that does not require antibodies and uses proteolytic digestion to yield readily ionizable proteotypic peptides that enables the sensitive, specific, and simultaneous quantitation of insulin and C-peptide.
Methods: Serum samples were precipitated with acetonitrile. Analytes were enriched using solid phase extraction and then digested with endoproteinase Glu-C. Surrogate peptides for insulin and C-peptide were analyzed using targeted LC-MS/MS.
Results: Inter-day imprecision was below 20 %CV and linearity was observed down to the lower limit of quantitation for both analytes (insulin = 0.09 ng/mL, C-peptide = 0.06 ng/mL). Comparison to a commercially available insulin immunoassay (Beckman Coulter UniCel DxI 600 Access) revealed a 30% bias between methods.
Conclusion: A novel LC-MS/MS method for the simultaneous analysis of insulin and C-peptide using Glu-C digestion was developed and evaluated. A detailed standard operating procedure is provided to help facilitate implementation in other laboratories.

1. Introduction

Diabetes is caused by a deficiency of insulin or by the inability to respond metabolically to the presence of insulin. As a peptide hormone, insulin is composed of a 21-amino acid A-chain connected via disulfide bonds to a 30-amino acid B-chain [1]. It is derived from its prohormone proinsulin, which is processed in the Golgi apparatus by prohormone convertases and carboxypeptidase E. During proinsulin processing, a 35-amino acid connecting peptide (C-peptide) is liberated and stored along with insulin in secretory granules that fuse with the plasma membrane and release insulin and C-peptide in response to increased blood glucose concentrations.

C-peptide's half-life in circulation is 6–10 times greater than insulin [2,3]. As a result, C-peptide is generally more abundant than insulin and, therefore, easier to accurately quantify. For this reason, direct insulin quantification is frequently forgone in favor of C-peptide measurement, which can be used to guide diagnosis, monitoring, and treatment. However, there are several circumstances in which a specific evaluation

of insulin itself is valuable. For example, the measurement of both analytes is necessary for studies of insulin production and clearance rates. Similarly, cases of factitious, accidental, or iatrogenic insulin overdosing can be more easily distinguished from disorders, such as insulinoma, when measurements of both insulin and C-peptide are available [4].

Many methods for detecting insulin currently exist, including automated immunoassays, ELISA-based assays, and liquid chromatography-mass spectrometry-based approaches [5]. Though these methods each have advantages, concordance among the methods is lacking, and a reference method for the measurement of insulin is desirable [6]. Insulin assays that rely on antibodies for detection can suffer from non-specificity due to antibody cross-reactivity with proinsulin or partially processed forms of proinsulin [7] and from insulin autoantibodies in many diabetic patients [8–10]. Mass spectrometry is particularly appealing for insulin detection due to its capacity to distinguish insulin, proinsulin, C-peptide, and many insulin analogs, as well as its potential for multiplexing [11,12].

We recently developed and validated an assay for C-peptide that uses liquid chromatography-tandem mass spectrometry (LC-MS/MS) [13]. In contrast to existing methods [12,14–16], the protocol used standard chromatography and did not require immunoaffinity enrichment to enhance the signal of the analyte. Instead, endoproteinase Glu-C was used to digest C-peptide into readily ionizable peptides, which were analyzed using tandem mass spectrometry. In the current study, we have developed and evaluated a modified version of that method, which is capable of quantifying insulin and C-peptide simultaneously.

2. Materials and methods

A detailed Standard Operating Procedure for the simultaneous quantification of insulin and C-peptide is presented in the Supplemental Material. A brief description of the method is provided here.

2.1. Description of samples

De-identified leftover clinical samples (serum) were obtained from the Clinical Chemistry Laboratory at the University of Washington Medical Center. This use of de-identified leftover samples for method development was reviewed by the Human Subjects Division of the University of Washington and determined to be non-human subjects-research (STUDY000011691).

2.2. Sample preparation

Samples (200 µL) and internal standards (C-peptide ^{13}C-labeled at two leucines and universally ^{15}N-labeled insulin) were precipitated with acetonitrile and centrifuged. The resulting supernatant was filtered (Pall) and dried using centrifugal evaporation. Analytes were reconstituted, acidified, and enriched using mixed anion exchange solid-phase extraction with a µ-elution plate (Waters). The eluate was dried using evaporation (Turbo-Vap, Biotage). The analytes were then reconstituted, reduced with dithiothreitol, alkylated with iodoacetamide, and proteolyzed with Glu-C (Sigma). The reaction was stopped with formic acid. During method development, it was observed that insulin adsorbed to both glass and plastic vessels in standard buffers. After testing a variety of conditions, we found that both analytes were stable in 0.001% Zwittergent 3–16 detergent in certain polypropylene tubes (Eppendorf Protein LoBind).

2.3. LC-Ms/MS

A Waters Acquity UPLC-Xevo TQ-S platform was used for LC-MS/MS analysis. Mobile phase A consisted of 0.1% formic acid and 2% dimethyl sulfoxide in water. Mobile phase B consisted of 0.1% formic acid and 2% dimethyl sulfoxide in methanol. Analytes were resolved with an Acquity

UPLC HSS T3 analytical column fitted with a Vanguard Pre-Column. Two peptides were monitored for native and isotopically-labeled C-peptide: EAEDLQVGQVE (proteotypic fragment used for quantitation) and LGGGPGAGSLQPLALE (for quality assurance; underlined leucines were ^{13}C-labeled in the internal standard). Two insulin B-chain peptides were monitored for native and isotopically-labeled insulin: RGFFYTPKT (proteotypic fragment used for quantitation) and FVNQHLCGSHLVE (for quality assurance). Product ion spectra are included in Supplemental Fig. 1. The assay was calibrated using a six-point calibration curve, which was made by spiking insulin and C-peptide certified reference materials into horse serum, which lacked the quantitative peptides.

2.4. Data analysis

Results were processed using Skyline [17,18] and Microsoft Excel. Product ion spectra were visualized with MassLynx (Waters Corporation). Concentrations were calculated using the slope of the calibration curves for insulin and C-peptide using peak area ratios and applying a linear fit with unspiked horse serum serving as zero. Through validation, assay and sample acceptance criteria were established: for each batch, the calibration regression coefficient r^2 was required to be > 0.98 and for each sample, the quantitative peptide internal standard peak area for each analyte was required to be > 50% of the mean observed for each batch.

2.5. Method evaluation

Evaluation included an assessment of imprecision, linearity, lower limit of quantification, analyte stability, the effects of common laboratory interferences, spike-recovery, and method comparison. Throughout the evaluation, system suitability was assessed each day using a pooled processed sample with a low concentration of heavy-labeled C-peptide internal standard material. All statistical analyses were performed in Excel.

2.5.1. Lower limit of quantitation

The lower limit of quantification (LLOQ) of the insulin assay was estimated by analyzing a human serum pool [with an average value of 0.31 ng/mL (53 pM) insulin] diluted with equine serum (negative for peptides of interest) to 75:25, 50:50, 25:75, 20:80, and 10:90 ratios of patient:equine sera in quintuplicate. Plots of %CV versus concentration were then fitted with a power curve and the LLOQ at 20% CV was calculated. One data point was omitted from the analysis because the internal standard peak area did not meet quality assurance acceptance criteria.

2.5.2. Imprecision

To assess imprecision, we performed a 5x5 study by analyzing five replicates of each of two pools of human serum with low [0.36 ng/mL (62 pM), 1.28 ng/mL] and high [11.30 ng/mL (1,946 pM), 12.22 ng/mL] concentrations of insulin and C-peptide, respectively, on each of five days. High analyte concentrations were achieved by spiking pooled human serum with insulin and C-peptide.

2.5.3. Linearity

Linearity of the assay was evaluated with an 11-point mixing study. Two separate pools were created from leftover clinical samples. One pool contained 0.36 ng/mL (62 pM) and 1.53 ng/mL of insulin and C-peptide, respectively. The other pool was spiked to 9.66 ng/mL (1,663 pM) and 8.31 ng/mL of insulin and C-peptide, respectively, using certified reference materials. The spiked and unspiked pools were mixed in 10% increments with one another and analyzed in triplicate. The coefficient of determination (r^2) and bias for the middle nine samples were determined.

2.5.4. Stability

Analyte stability in serum and in prepared samples at low [0.41 ng/mL (71 pM), 1.55 ng/mL] and high [10.83 ng/mL (1,865 pM), 8.70 ng/mL] concentrations of insulin and C-peptide, respectively, were assessed by comparing results of treated samples to untreated control samples in triplicate. The conditions assessed for serum samples included storage at room temperature (4 h), 4 °C (24 h), or exposure to one or two freeze–thaw cycles prior to preparation and analysis. Stability was assessed in prepared samples after storage in the autosampler of the LC system at 5 °C (24 h), in the freezer at −80 °C (72 h), or after exposure to one or two freeze–thaw cycles. Bias was calculated relative to unstressed samples.

2.5.5. Spike recovery

Horse serum samples were spiked to 1, 5, or 10 ng/mL with both C-peptide and insulin certified reference materials (N = 5). Similarly, five individual human serum samples were spiked to 5 ng/mL. The percent recovery of each human subject sample was calculated relative to the recovery observed for horse serum spiked to 5 ng/mL.

2.5.6. Interference and sample type

The effects on the assay of common clinical conditions (liver disease resulting in hyperbilirubinemia, hypertriglyceridemia, kidney disease resulting in uremia and elevated creatinine, and hyperproteinemia) were evaluated using leftover clinical samples spiked with either buffer or 6.43 ng/mL of insulin and C-peptide certified reference materials. The recovery in each clinical sample that contained a high level of potential interference was compared to the recovery in a normal control human serum pool spiked in a similar manner. The effect of hemolysis was assessed using a kit from Sun Diagnostics (Int-01). A normal human serum pool was spiked to achieve high hemoglobin levels, which were subsequently quantified using a Radiometer ABL90 Flex Plus blood gas analyzer. Two samples with insulin autoantibodies (IAA indices of 1.344 and 3.35) [19] were spiked to 6.43 ng/mL insulin and C-peptide and analyzed before and after spiking. Generic lispro and glargine were obtained from the pharmacy at the University of Washington Medical Center and were spiked into two pools of normal human serum at 10 ng/mL. Proinsulin (Amidebio) was spiked into separate aliquots of the same pools of normal human serum at 200 pM. To assess whether EDTA plasma was an acceptable sample type, leftover purple top tubes (BD) were spiked with either buffer or 6.43 ng/mL insulin and C-peptide certified reference materials and compared to gold top serum controls.

2.5.7. Method comparison

The insulin LC-MS/MS assay was compared to a commercially available automated clinical immunoassay (Beckman Coulter UniCel DxI 800). Results were correlated and %Bias was calculated.

3. Results

3.1. Method development

The goal of this work was to multiplex insulin into our clinically validated assay for C-peptide with minimal changes to the overall protocol and no impact on the quantification of C-peptide [13]. Several Glu-C peptides from insulin were optimized and evaluated for favorable chromatographic performance. Transitions that were free from interference in both human and horse serum were selected, however there was only one proteotypic peptide that could distinguish insulin from proinsulin, which was the C-terminal B chain peptide RGFFYTPKT. Ultimately, both RGFFYTPKT (quantitative peptide) and the N-terminal B chain peptide FVNQHLCGSHLVE (for quality assurance) were carried forward. A digestion time course experiment revealed that endoproteinase Glu-C digested both C-peptide and insulin efficiently in serum samples using the established C-peptide assay sample preparation method (Supplemental Fig. 2). The proteolytic release of the two insulin

peptides appeared to reach a plateau within 90 min. Ion suppression from serum matrix was observed for the quantitative peptide from insulin (~26%), but no ion suppression was observed for C-peptide (Supplemental Fig. 3).

3.2. Method evaluation

The method was evaluated for imprecision, linearity, lower limit of quantification, analyte stability, the effects of common laboratory interferences, spike recovery, and method comparison.

3.2.1. Lower limit of quantification

The LLOQ for insulin was estimated by fitting a power curve to a plot comparing the imprecision (%CV) of six low-concentration samples [insulin concentration ranged from 0.032 to 0.32 ng/mL (5.5–55 pM)] against the expected concentration for each sample and solving for the concentration at which %CV = 20 (Fig. 1). Using this method, the lower limit of quantification was estimated to be 0.09 ng/mL (15 pM) for insulin. C-peptide, which ranged from 0.135 to 1.35 ng/mL, was also evaluated in this experiment and was linear over the range, consistent with the original LLOQ determination of 0.06 ng/mL (Supplemental Fig. 4) [13].

3.2.2. Imprecision

The imprecision of the assay was evaluated by analyzing human serum pools with low or high concentrations of insulin and C-peptide in five replicates on five different days. For insulin, the within-batch imprecision (%CV) for the low concentration sample [0.36 ng/mL (62 pM) insulin] ranged from 4.5 to 11.9% and for the spiked high concentration sample [11.3 ng/mL (1,946 pM) insulin] from 0.7 to 7.8% (Table 1). For C-peptide, the within-batch imprecision for the low concentration sample (1.28 ng/mL C-peptide) ranged from 1.6 to 10.5%, and for the high concentration sample (12.2 ng/mL C-peptide) from 3.0 to 4.1% (Table 2). Inter-day imprecision (sum of squares) in low and high concentration human serum pools was 18.2% and 10.3%, respectively, for insulin and 10.5% and 8.7%, respectively, for C-peptide. These results are in agreement with the total imprecision of 7.7% determined for C-peptide in the original assay [13].

3.2.3. Linearity

The linearity of the assay was evaluated using an 11-point mixing series (Fig. 2). Insulin was linear between 0.09 and 9.66 ng/mL (15–1,663 pM), with a slope of 1.03, a coefficient of determination r^2 of 0.991, and bias from expected ranging from −11.7 to 5.9%. One outlier was removed from analysis at the 70% high pool level after visual inspection per CLSI guideline EP06 (Supplemental Fig. 5) [20]. With the outlier included, the slope was 1.04 and r^2 was 0.977. For C-peptide, linearity was reaffirmed between 1.53 ng/mL and 8.31 ng/mL, the slope was 1.00, r^2 was 0.993, and bias from expected ranged from −7.3 to 3.5%.

3.2.4. Stability

The stability of insulin and C-peptide before and after sample preparation was assessed by subjecting samples to a variety of conditions and determining the bias compared to unstressed samples (Supplemental Tables 1 and 2). For samples held for 4 h at room temperature, 24 h at 4 °C, or subjected to one or two freeze–thaw cycles prior to sample preparation, the mean observed bias was < 20% for insulin and C-peptide. Likewise, the mean bias observed for prepared samples that had been held for 24 h at 5 °C, >72 h at −80 °C, or subjected to one or two freeze–thaw cycles was also < 20%, except for insulin after 24 h in the refrigerated autosampler, which exhibited a significant bias (−21.1%).

3.2.5. Interference and tube type

To assess the effects of common clinical interferences, recovery of spiked C-peptide and insulin was compared in leftover clinical samples
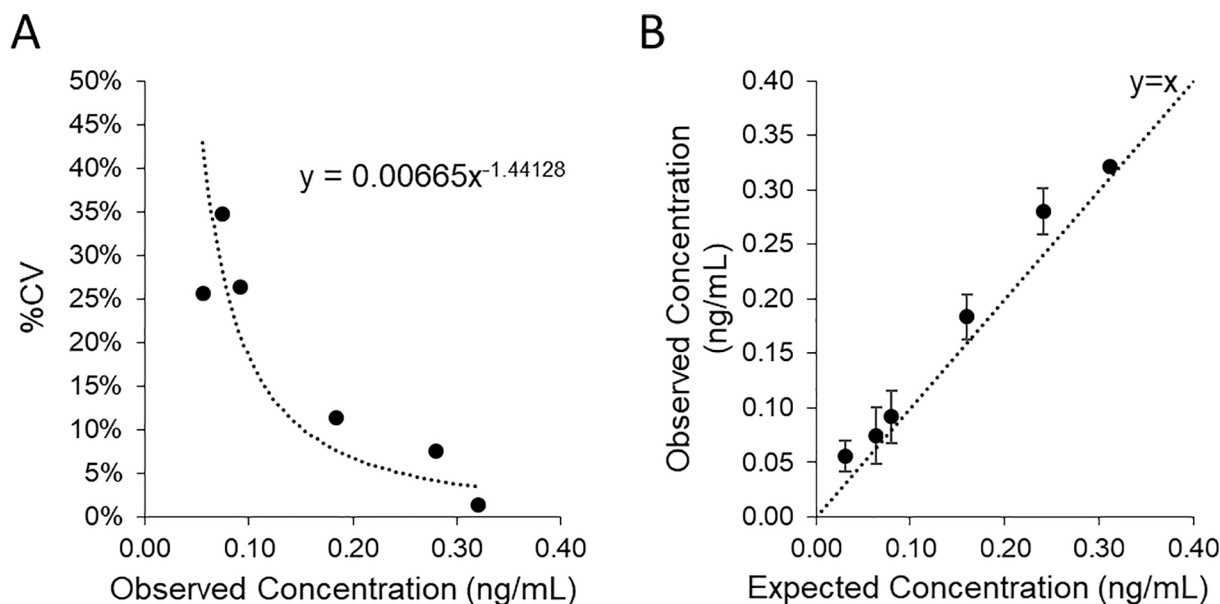
Fig. 1. Insulin lower limit of quantitation. Five replicates of six different samples were analyzed in one day. (**A**) A power function was fit to a plot of the imprecision (%CV) vs. the expected concentration of each dilution. (**B**) A plot of the observed vs. expected concentration for each dilution is illustrated. Residuals from y = x were < 20%, error bars denote SD. Concentrations of insulin in ng/mL can be converted to pM by multiplying by 172.18.

Table 1
Insulin 5 × 5 Imprecision Study.[a]

Low Pool	Insulin Concentration (ng/mL) Replicate					Within-day CV
Day	1	2	3	4	5	
1	0.39	0.36	0.45	0.40	0.36	9.4%
2	0.40	0.46	0.38	0.46	0.36	11.3%
3	0.34	0.37	0.02[c]	0.37	0.37	4.5%
4	0.24	0.30	0.34	0.31	0.29	11.9%
5	0.35	0.30	0.34	0.29	0.36	9.5%
Between-day CV	17.8%	18.4%	13.8%	18.5%	10.0%	Total Imprecision[b] 18.2%
High Pool	**Insulin Concentration (ng/mL) Replicate**					**Within-day CV**
Day	**1**	**2**	**3**	**4**	**5**	
1	12.1	12.1	12.1	11.9	12.0	0.7%
2	11.6	11.7	12.3	12.2	12.1	2.5%
3	10.1	10.4	10.7	10.5	10.0	2.7%
4	10.0	9.7	10.3	9.78	9.81	2.3%
5	11.3	11.4	11.8	13.3	13.2[c]	7.8%
Between-day CV	8.5%	8.8%	7.9%	12.3%	11.2%	Total Imprecision[b] 10.3%

[a] Five replicates of the same pool of human serum were analyzed on each of five days. The high pool was spiked with insulin to achieve the high concentrations. Concentrations in ng/mL can be converted to pM by multiplying by 172.18.
[b] Total imprecision was estimated using the sum of squares: $[(\text{within-day CV})^2 + (\text{between-day CV})^2]^{0.5}$.
[c] Replicate was discarded due to its internal standard falling below assay quality assurance requirements.

Table 2
C-peptide 5 × 5 Imprecision Study.[a]

Low Pool	C-peptide Concentration (ng/mL) Replicate					Within-day CV
Day	1	2	3	4	5	
1	1.42	1.35	1.43	1.38	1.48	3.6%
2	1.21	1.13	1.17	1.14	1.17	2.7%
3	1.33	1.15	1.82[c]	1.27	1.05	10.5%
4	1.26	1.28	1.29	1.32	1.30	1.6%
5	1.45	1.18	1.43	1.28	1.35	8.5%
Between-day CV	7.8%	7.8%	9.3%	7.0%	13.1%	Total Imprecision[b] 10.5%
High Pool	**C-peptide Concentration (ng/mL) Replicate**					**Within-day CV**
Day	**1**	**2**	**3**	**4**	**5**	
1	13.3	12.3	13.5	13.0	13.0	3.6%
2	11.4	10.8	11.7	11.5	11.0	3.2%
3	11.1	11.0	11.4	11.7	10.9	3.0%
4	12.0	12.3	12.7	12.0	13.1	4.1%
5	12.5	13.0	13.2	13.5	13.5[c]	3.1%
Between-day CV	7.3%	7.9%	7.4%	7.1%	10.1%	Total Imprecision[b] 8.7%

[a] Five replicates of the same pool of human serum were analyzed on each of five days. The high pool was spiked with C-peptide to achieve the high concentrations.
[b] Total imprecision was estimated using the sum of squares: $[(\text{within-day CV})^2 + (\text{between-day CV})^2]^{0.5}$.
[c] Replicate was discarded due to its internal standard falling below assay quality assurance requirements.

that did not have detectable amounts of known interferences to those that did. To evaluate for interference, we assessed whether there was a statistical association between recovery and increasing concentration of each interference and if the mean recovery was between 80 and 120% of expected. With these metrics as a guide, samples from patients with liver disease and bilirubin concentrations up to 38.7 mg/dL, uremic samples with creatinine concentrations up to 13.13 mg/dL, hemoglobin concentrations up to 1.3 g/dL, triglyceride concentrations up to 2,142 mg/dL, and total protein concentrations up to 9.1 g/dL did not substantially

interfere with the quantification of insulin (Fig. 3) or C-peptide (Fig. 4). Of note, hemolyzed samples showed reduced insulin internal standard peak areas (60–72%) compared to control (data not shown). This would suggest either matrix interference or insulin degradation by insulin-degrading enzyme, which is released on hemolysis [21–24]. Two samples with insulin autoantibodies spiked with insulin and C-peptide demonstrated recoveries of each analyte between 98 and 103% (data not shown).

It is possible for certain insulin analogs that are commonly used in clinical practice to interfere with insulin assays [25,26]. With respect to
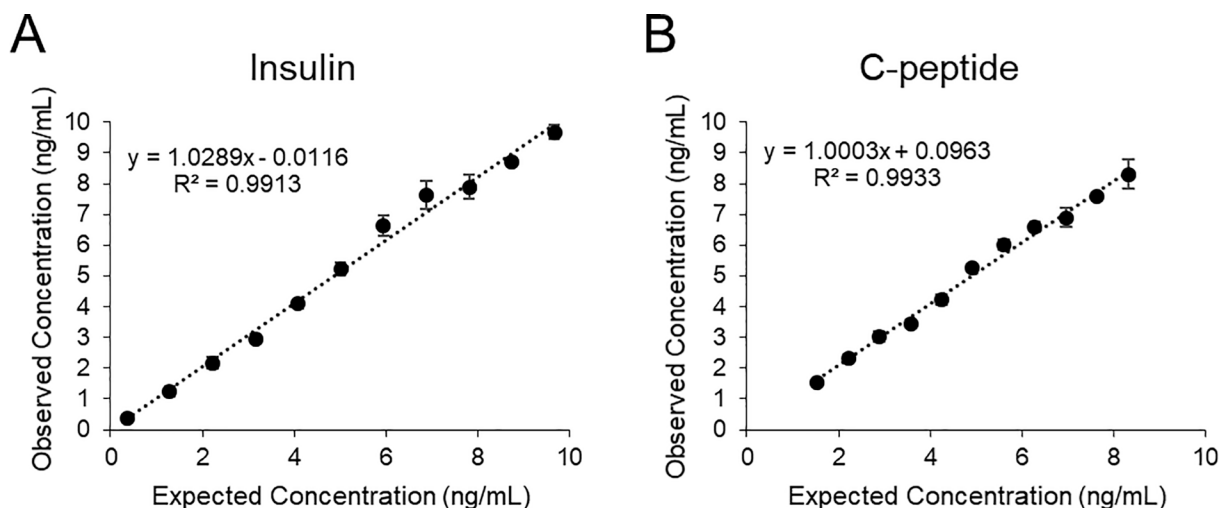
Fig. 2. Insulin and C-peptide Linearity. An 11-point mixing study was performed by mixing spiked and unspiked human serum pools in 10% increments. Samples were analyzed in triplicate. One sample was removed from the insulin analysis as an outlier per CLSI EP06 guideline. Concentrations of insulin in ng/mL can be converted to pM by multiplying by 172.18.
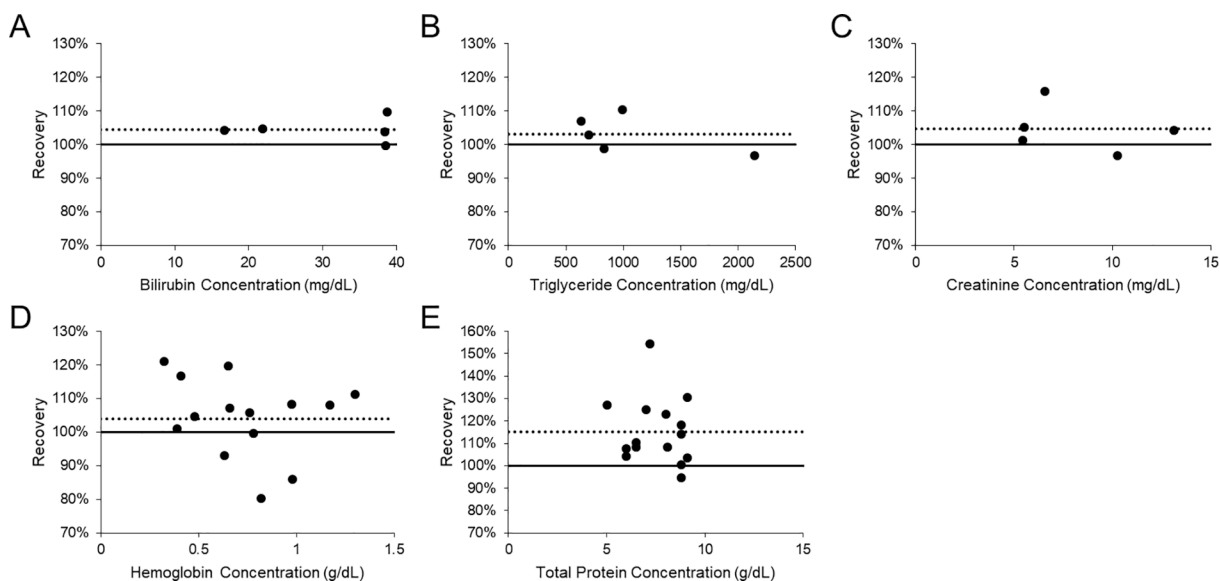

Fig. 3. Effects of common laboratory interferences on insulin quantitation. Samples containing high concentrations of potential interferences were spiked with insulin and the recovery was compared to that of healthy control samples. Dotted lines show average recovery for samples with elevated (**A**) bilirubin 104%, (**B**) triglycerides 103%, (**C**) creatinine 105%, (**D**) hemolysate 104% (**E**), and total protein 115%.

this new assay for insulin, the surrogate peptide from each analog that corresponds to the quantitative peptide in insulin (RGFFYTPKT) has a different precursor mass (Supplemental Table 3), except for lispro (RGFFYTKPT). There was no signal detected for insulin when lispro was spiked into normal human serum. The corresponding surrogate peptide of glargine (RGFFYTPKTRR) has a different precursor mass, but its main circulating metabolite (M1) is produced by peptidase cleavage of the C-terminal arginine residues, which would yield a surrogate peptide identical to that of insulin. Spiking glargine to 10 ng/mL in normal human serum led to the detection of 5.6 ng/mL of insulin in the new assay, suggesting that the two arginine residues from ~ 56% of glargine were removed when spiked into the serum pools. The possible interference of proinsulin in our assay was similarly evaluated. Insulin and C-peptide were not detectable at a spike level of 200 pM proinsulin (the top of the physiological range for proinsulin, data not shown).

To assess whether EDTA plasma was an acceptable sample type for the assay, the recovery of C-peptide and insulin spiked into EDTA

plasma (purple top) was compared to analyte spiked into control serum samples (gold top). Recoveries in human plasma samples of five individual subject samples showed no significant bias compared to control serum, with observed average recoveries for insulin and C-peptide of 102% and 95%, respectively (Supplemental Fig. 6).

3.2.6. Spike-recovery

Spike-recovery of insulin and C-peptide in horse and human serum was also evaluated (Fig. 5). Recovery ranged 97–100% for insulin and 79–108% for C-peptide for five different human serum samples relative to horse serum.

3.2.7. Method comparison

The performance of the new LC-MS/MS assay for insulin was also compared with a commercially available immunoassay (Beckman Coulter UniCel DxI 800) using leftover clinical samples (Fig. 6). Linear regression demonstrated a coefficient of determination of $r^2 = 0.95$. The
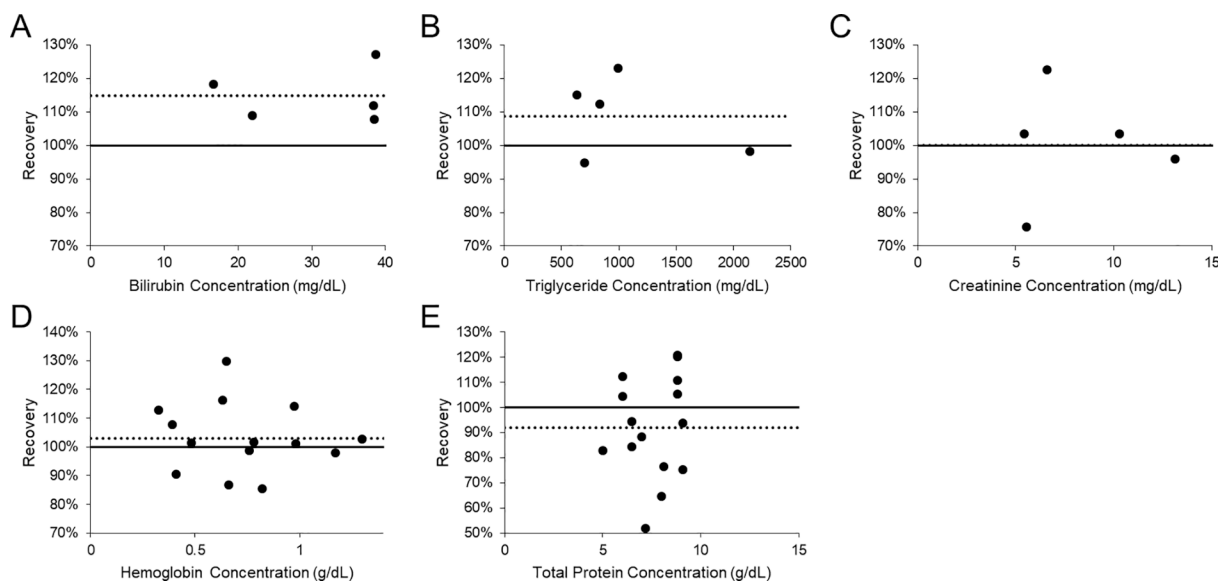
Fig. 4. Effects of common laboratory interferences on C-peptide quantitation. Samples containing high concentrations of interferences were spiked with C-peptide and the recovery was compared to that of healthy control samples. Dotted lines show average recovery for samples with elevated (**A**) bilirubin 115%, (**B**) triglycerides 109%, (**C**) creatinine 100%, (**D**) hemolysis 103%, and (**E**) total protein 92%.

Sample	Slope	Intercept	% Recovery
Subject 1	0.1584	0.059	97
Subject 2	0.1596	0.1407	98
Subject 3	0.1630	0.2048	100
Subject 4	0.1566	0.0328	96
Subject 5	0.1608	0.3663	99
Horse serum	0.1638	0.0244	N/A

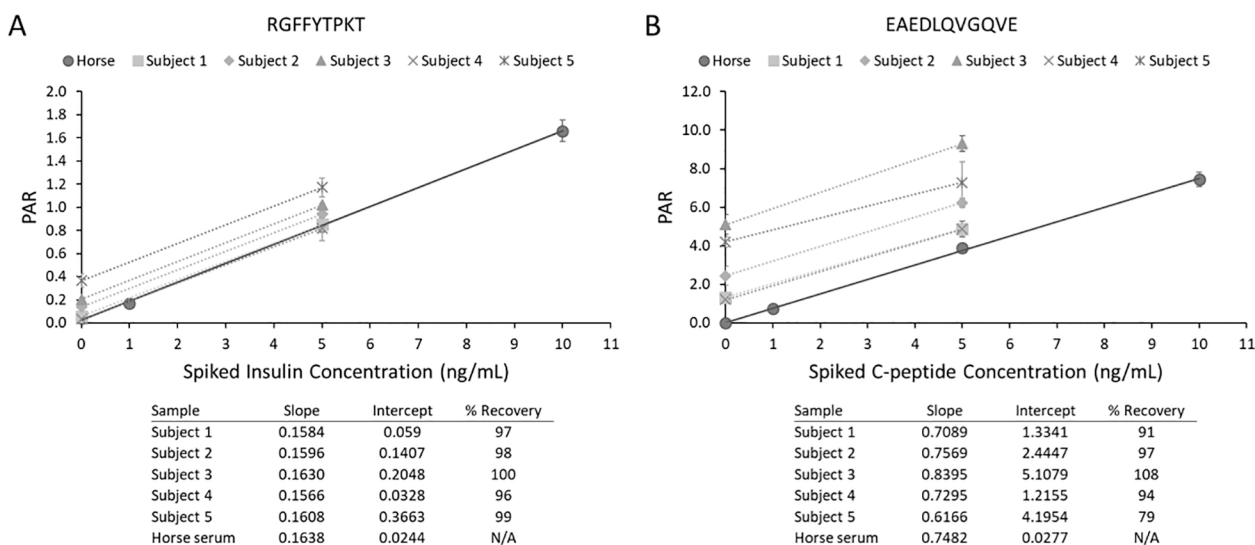| Sample	Slope	Intercept	% Recovery
Subject 1	0.7089	1.3341	91
Subject 2	0.7569	2.4447	97
Subject 3	0.8395	5.1079	108
Subject 4	0.7295	1.2155	94
Subject 5	0.6166	4.1954	79
Horse serum	0.7482	0.0277	N/A

Fig. 5. Spike recovery in human samples relative to horse serum. Observed peak area ratio (PAR) is plotted vs. the concentration of spiked insulin (**A**) and C-peptide (**B**) for five human subjects (three replicates) and horse serum (five replicates). Data are presented as the mean and standard deviation. The slopes, intercepts, and % recovery (calculated relative to horse serum spiked to 5 ng/mL) are displayed below the graphs. Concentrations of insulin in ng/mL can be converted to pM by multiplying by 172.18.

mean relative difference across all samples between the LC-MS/MS assay and the immunoassay was 29.5%, with the LC-MS/MS assay reporting higher values. The horse serum-based calibrators developed in the new assay were also run by the immunoassay and did not explain the observed bias. More specifically, linear regression of the concentrations observed by immunoassay vs. the expected concentrations (based on gravimetric preparation of the calibrators) was Observed = 1.03 * Expected + 0.40, which would predict that the LC-MS/MS assay would report similar results to the immunoassay.

4. Discussion

Most previously described LC-MS/MS methods for the quantification of insulin and C-peptide have relied on measuring intact proteins, which ionize somewhat poorly using electrospray ionization. To achieve the desired sensitivity, these methods have relied upon 2-dimensional chromatography [11,14,16], immunoaffinity enrichment [14,15,27], and high resolution mass spectrometers [12], which can be more difficult to adapt to a high-throughput clinical laboratory setting or, in the case of immunoaffinity enrichment, have the potential to suffer from autoantibody interference. Our new method provides the simultaneous quantification of insulin and C-peptide without the need for these techniques by instead digesting insulin and C-peptide into readily ionizable proteotypic peptides using Glu-C. This enzyme was found to robustly digest both analytes in a serum extract within 2 h. The method is quite sensitive, with LLOQ of 0.09 ng/mL (15 pM), and is free from interference from proinsulin and insulin autoantibodies, which could be useful in the accuracy assessment or standardization of other assays in the future.

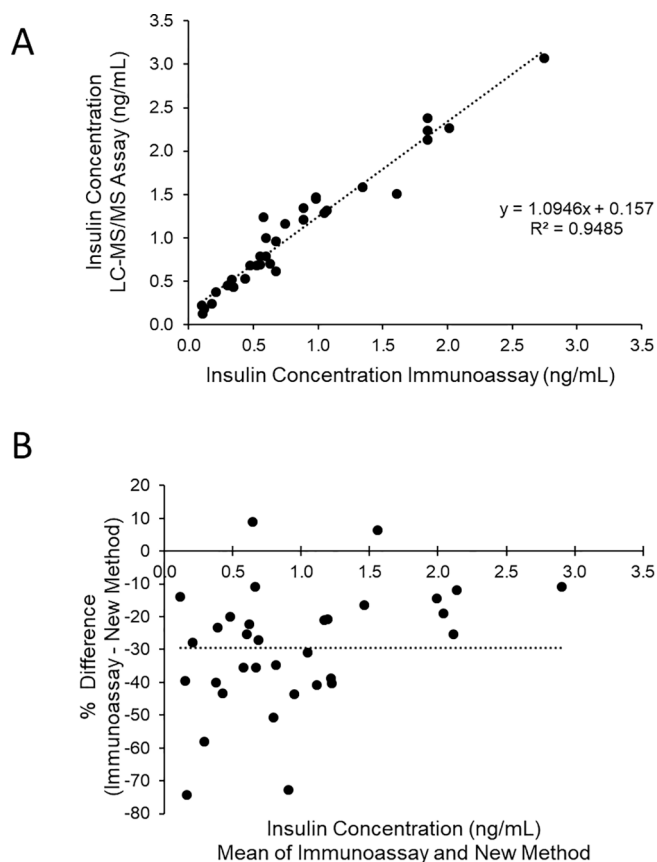During method development, we evaluated for matrix interference

Fig. 6. Insulin method comparison. The concentrations of insulin in 33 human samples were measured with a commercially available immunoassay (Beckman Coulter UniCel DxI 800) and by the new LC-MS/MS assay. (**A**) The concentration observed by LC-MS/MS is plotted vs. the concentration observed by immunoassay. The results of linear regression analysis are shown. (**B**) The percent difference between immunoassay and LC-MS/MS assay measurements is plotted vs. the mean of the two measurements. The dashed line illustrates the mean bias of 29.5%. Concentrations of insulin in ng/mL can be converted to pM by multiplying by 172.18.

and found that the signal from insulin peptides was much lower in solvents containing only methanol and water compared with either serum digest or solvents containing bovine serum albumin. From these data, we hypothesize that insulin adsorbed to its polypropylene or glass vessel when dissolved in solvents lacking carrier protein or peptide, which is consistent with other reports [14]. Importantly, we did not observe this effect with C-peptide. As a result of this problem, we carefully designed our standard operating procedure to minimize the amount of time that unlabeled or isotope-labeled insulin was diluted without carrier protein. In addition, the surrogate peptides for insulin were unstable in prepared samples when kept in refrigerated conditions, which was more significant for samples with low concentrations of insulin (0.41 ng/mL, −21.1% bias) compared to samples with high concentrations (10.83 ng/mL, −7.8% bias), which is consistent with what has been observed for full-length insulin in previous publications [28,29].

The mean observed bias due to common laboratory interferences (bilirubin, triglyceride, uremia, hemoglobin, and total protein) was < 20%. Insulin autoantibodies and proinsulin also did not interfere. However, analysis of hemolyzed samples could be problematic due to the release of insulin degrading enzyme from red blood cells [21–24]. We did observe decreased internal standard peak areas in hemolyzed samples, which could be explained by this enzymatic activity, although interference due to the elevated protein concentration cannot be ruled out. In addition, our experiments to test the influence of total protein on the assay led to an acceptable mean bias, but the recovery results were

more variable than for other interferences. The reason for this is unclear, but it is in contrast with our previous results for C-peptide [13].

We evaluated the possible influence of insulin analogs on the accuracy of the assay. Because we proteolyze insulin and its analogs into shorter peptides with GluC, there is the potential for a loss of specificity that is maintained with intact protein analysis. Most of the precursor masses of the surrogate peptides liberated from human insulin analogs are different from human insulin (Supplemental Table 3). However, the surrogate peptide from lispro is identical in mass. We hypothesized that the inversion of the lysine and proline in the sequence would make the formation of the b_6 fragment ion much less favorable, since gas-phase fragmentation on the amino-terminal side of proline is preferred. Indeed, we observed no interference when spiking lispro into the assay. Another analog that could potentially interfere is glargine. While the precursor mass of the surrogate peptide from intact glargine is different from human insulin, it is well known that glargine is rapidly metabolized to its predominant M1 metabolite, which is lacking the carboxyl-terminal arginine residues [30]. When we spiked glargine into serum, we observed a significant signal for insulin, confirming the rapid conversion of glargine *ex vivo* due to active peptidases in serum. As a result of this interference, results from patients and research participants who are using glargine for glucose control will have concentrations of insulin detected in this assay above what is actually being produced endogenously.

In our method comparison, we observed a substantial mean bias of 29.5% between the LC-MS/MS assay and the Beckman immunoassay. Such a difference is remarkable, but perhaps not surprising. Previous studies have demonstrated that insulin quantification by different immunoassay instruments can vary by as much as 66% [6]. Inherent issues with insulin stability, multimerization, and adsorption make the formulation of accurate insulin calibrators challenging, and there is a great need for standardization across assays [31].

We have provided a detailed standard operating procedure to facilitate implementation of the method in other laboratories. In addition, interlaboratory comparisons of the method are ongoing as part of the Targeted Mass Spectrometry Assays for Diabetes and Obesity Research (TaMADOR) consortium. Goals of the consortium include the validation of this assay at other sites, standardization of calibrators and control materials, and the verification of other LC-MS/MS instruments (https://panoramaweb.org/tamador.url).

5. Conclusion

A novel multiplexed LC-MS/MS method for quantitation of insulin and C-peptide in human serum and plasma was developed and evaluated. A detailed standard operating procedure is provided to facilitate implementation in other laboratories.

Declaration of Competing Interest

The 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.2022.06.003.

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