Quantitative Insulin Analysis Using Liquid Chromatography–Tandem Mass Spectrometry in a High-Throughput Clinical Laboratory

Zhaohui Chen, Michael P. Caulfield, Michael J. McPhaul, Richard E. Reitz, Steven W. Taylor, and Nigel J. Clarke*

BACKGROUND: Circulating insulin concentrations reflect the amount of endogenous insulin produced by the pancreas and can be monitored to check for insulin resistance. Insulin is commonly measured using immunochemiluminometric assays (ICMA). Unfortunately, differing crossreactivities of the various ICMA antibodies have led to variability in assay results. In contrast, liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based approaches can provide a highly specific alternative to immunoassays.

METHODS: Insulin was extracted from patient serum and reduced to liberate the insulin B chain. Subsequent resolution of the peptide was achieved by LC coupled to triple-quadrupole MS. Selected-reaction monitoring of B-chain transitions was used for quantification. Recombinant human insulin was used as a calibrator and was compared as internal standards. Bovine insulin and a stable isotopic-labeled (13C/15N) human insulin B chain were used and compared as internal standards.

RESULTS: The LC-MS/MS assay described herein has been validated according to CLIA guidelines with a limit of detection of 1.8 μIU/mL (10.8 pmol/L) and a limit of quantitation of 3 μIU/mL (18.0 pmol/L). A correlation between the LC-MS/MS assay and a US Food and Drug Administration-approved ICMA was completed for patient samples and the resulting Deming regression revealed good agreement. A reference interval for the assay was established.

CONCLUSIONS: A simple, high-throughput, quantitative LC-MS/MS insulin assay traceable to the NIBSC standard has been successfully developed and validated.

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Nonstandard abbreviations: ICMA, immunochemiluminometric assays; MS, mass spectrometry; SRM, selected reaction monitoring; LC-MS/MS, liquid chromatography–tandem MS; TCEP, tris-(2-carboxyethyl) phosphine; NIBSC, National Institute for Biological Standards and Control; IS, internal standard; FDA, US Food and Drug Administration; SPE, solid-phase extraction; LOQ, limit of detection; LOD, limit of quantitation.

1 Quest Diagnostics Nichols Institute, San Juan Capistrano, CA.
2 Address correspondence to this author at: Quest Diagnostics Nichols Institute, 33068 Ortega Hwy, San Juan Capistrano, CA, 92675. Fax 949-728-4872; e-mail Nigel.J.Clarke@questdiagnostics.com.

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more, auto- or heterophilic antibodies present within the patient’s sample may cause biases in reported insulin concentrations.

Mass spectrometry (MS)-based protein/peptide assays have recently been developed as important alternative strategies for clinical analysis of biomolecules (8), including insulin. Because the insulin is present in human blood in very low concentrations, most previously developed MS methods for the polypeptide’s detection and quantification have been dependent on immunological capture methodologies (9–15). However, recent improvements in the sensitivity of MS negate the requirement of antibody-based enrichment without compromising high specificity. Accordingly, the approach we have developed requires no capture antibody while still providing a highly specific and sensitive assay for human insulin through selected-reaction monitoring (SRM) analysis on a triple-quadrupole mass spectrometer.

Mature human insulin (molecular weight, 5808 Da) consists of 2 peptide chains (A and B) linked by 2 disulfide bonds. The insulin A (molecular weight, 2377 Da) and B chains (molecular weight, 3431 Da) can be separated using suitable reducing agents. The insulin B chain has been studied extensively; however, a quantitative assay for the peptide has not been developed (12, 16, 17). Our aim was to develop an assay for quantification of total insulin concentrations in human serum via quantifying the B chain after reduction and liberation from the intact molecule. Herein, we report the validation of this assay and reference interval studies to support the high-throughput clinical measurement of insulin by liquid chromatography–tandem MS (LC-MS/MS).

Materials and Methods

STANDARDS AND REAGENTS

HPLC-grade acetonitrile, isopropanol, ethanol, acetone, and water were from Burdick and Jackson; methanol and bond-breaker tris-(2-carboxyethyl) phosphine (TCEP) solution were from Thermo Fisher Scientific. High-purity formic acid was from Fluka. WHO human insulin standard (code: 66/304) was from the National Institute for Biological Standards and Research (NIBSC). Recombinant human insulin was from Millipore. Bovine insulin (>95% purity) and 1.5 mol/L Tris (Trizma) base solution were from Sigma. Fully synthetic, isotopically labeled insulin B chain was from Anaspec (see Fig. 1 in the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue9). Hypo-OptiClear stripped human serum free of insulin was from Biocell laboratories. All protein components were fully characterized by gel electrophoresis, HPLC, and MS by the manufacturers. Amino acid analysis was performed by AAA Service Laboratory Inc. to confirm protein content before use.

PREPARATION OF CALIBRATORS, CONTROLS, AND INTERNAL STANDARDS

A stock solution of human insulin standard (recombinant human insulin unless otherwise stated) at 1 IU/mL (6 μmol/L) was prepared in 0.2% formic acid solvent and stored at −80 °C until use. Immediately before assay, the stock solution was thawed and an aliquot diluted into insulin-free human stripped serum at a final concentration of 4000 μIU/mL (24 nmol/L). Dilutions were subsequently performed to generate a calibration series at concentrations of 5.0, 10, 15, 25, 50, 100, 200, and 300 μIU/mL (30.0–1800 pmol/L).

In-house QC stocks were prepared using recombinant human insulin from Millipore, dissolved in 0.2% formic acid and stored at −80 °C. The concentration of insulin in these stocks was verified by amino acid analysis before dilution. Five determinations were performed over a period of 6 months with a CV of 3%. QC controls (8, 16, 40, and 80 μIU/mL, or 48–480 pmol/L) were made by spiking Human Hypo-OptiClear stripped serum to the target concentrations and then storing them in aliquots at −80 °C. The newly made insulin QC controls and calibrators were assessed by comparison to NIBSC reference material. Each QC aliquot was used only once after thawing to avoid freeze–thaw cycles.

The internal standards (ISs) were prepared in 0.2% formic acid at a concentration of 10 μmol/L. Bovine insulin (350 pmol) was added to serum during the extraction (before reduction) and isotopically labeled human B chain (115 pmol) added to reduced extract before LC.

HUMAN STUDY PARTICIPANTS APPROVAL

Serum samples were obtained from healthy study participants following informed consent and stored at −80 °C until use (Western Institutional Review Board approval #1085473). The use of anonymized discarded samples in these studies was reviewed by the Western Institutional Review Board and deemed exempt.

CLINICAL SAMPLE COLLECTION

Unless otherwise stated, blood was collected into barrier-free serum preparation tubes (red top) and allowed to clot. The resulting serum was immediately processed then stored at −80 °C until analysis.

SAMPLE PREPARATION

Patient serum was thawed and vortex mixed, and 150 μL was vigorously mixed with 350 μL of basic ethanol (85% ethanol, 15% Tris base) and allowed to incubate for 60 min at −20 °C. The resulting precipitate was
pellet by centrifugation for 10 min at 5200g. Clarified supernatant (250 μL) was then mixed with 20 μL of bond-breaker TCEP solution (Thermo Fisher Scientific) which was used per the manufacturer’s directions to liberate the insulin A and B chains through reduction of their disulfide bonds.

AUTOMATED PREPARATIVE AND ANALYTICAL CHROMATOGRAPHY

Analytical separation of insulin B chain from matrix components before MS was achieved with a TurboFlow Aria TLX-4 (Thermo Fisher), a fully automated online LC system. The 4 columns in this LC system are operated in parallel, facilitating high throughput. The sample cleanup and enrichment was performed by online solid-phase extraction (SPE) using an Oasis HLB cartridge column (2.1 × 20 mm, 25 μm) (Waters) (see online Supplemental Fig. 2). Chromatographic resolution was accomplished using a Magic C4 column (2.1 × 50 mm, 5 μm, 300 Å) (Bruker- Michrom). For both SPE and analytical columns, we used the same solvent A (water, 0.2% formic acid) and B (acetonitrile, 0.2% formic acid). After injection of the extract (225 μL) at 4 mL/min, the cartridge was washed with 12% B solvent for a further 60 s. The analytes were then back-flushed off the extraction cartridge with a step gradient of 35% solvent B at 0.8 mL/min and refocused onto the analytical column, by means of a T valve with a 3:1 ratio of flow rates between eluting (0.6 mL/min) and loading (0.2 mL/min) pumps, respectively. Finally, the analytes were resolved using a fast, linear gradient from 12% to 42% solvent B at 0.5 mL/min over 3 min.

DATA ACQUISITION AND PROCESSING

A TSQ Vantage (ThermoFisher) triple quadrupole mass spectrometer interfaced to the TLX-4 system with an ESI probe served as the MS/MS detector. The data were acquired using SRM in positive ion mode under the following conditions: ionization voltage, 4800 V; sheath gas pressure, 50 (arbitrary units); auxiliary gas pressure, 25 (arbitrary units); capillary temperature, 250 °C. Instrument settings in Xcalibur included positive scan mode with the Chrom filter enabled and set at 1.5 mTorr; and both Q1 and Q3 peak widths set to 0.7.

The unique nature of the amino acid sequence for the insulin B chain was confirmed by running a BLAST (Basic Local Alignment Search Tool) search against the nonredundant database (18) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In this assay, we consider the retention time and the ratio of the mass transitions (1:1, with tolerance of ±2%) to support B-chain identification. For quantitative analysis, the most intense isotopic peak of the MH+ + i 3 ion of insulin chain B [m/z 686.9 (0.2)] was used as the precursor ion (see online Supplemental Fig. 3). Transitions to b14, + i 2 [768.5 (0.2)] and y13, + i 2 [753.2 (0.2)] from insulin B chain, with optimal collision energies of 21 and 19 V, respectively, were monitored by SRM to enhance selectivity. The corresponding transitions for the ISs were 680.8 (0.2) to 768.5 (0.2), 738.3 (0.2) for bovine insulin, and 688.1 (0.2) to 768.5 (0.2), 756.0 (0.2) for isotopically labeled human insulin B chain. To enhance sensitivity, the peak areas for each transition were summed for each peptide. The ratio of the peak area of the analyte to the IS (unless otherwise stated, isotopically labeled human insulin B chain was employed as the IS) was used to calculate the concentrations from the standard curve. A weighted linear model (1/x) was used for generation of the standard curve by linear regression. Results were reported as the concentration of insulin (μU/mL) or pmol/L; 1 μU/mL = 6 pmol/L). Software tools used were TSQ Vantage 2.0.0, Tune Master V 2.0.0, Xcalibur V 2.0.7, SP1, LC Quan V 2.5.6, SP1, and XReport 2.0.7. SP1 and ARIA OS V 1.6.1 (ThermoFisher) were used for all the data acquisition and processing.

METHOD COMPARISON

The LC-MS/MS method was compared to a US Food and Drug Administration (FDA)-approved commercial (Beckman Access® ICMA) platform [limit of detection (LOD) 0.13 μU/mL; limit of quantitation (LOQ) 0.3 μU/mL] for measurement of insulin in patients (n = 89). These patient samples were deidentified discards submitted previously for routine clinical testing. Concentrations measured covered the expected human insulin reference intervals and beyond from low to very high insulin concentrations. Correlation of the 2 methods was evaluated through the use of a Deming regression.

REFERENCE INTERVAL DETERMINATION

A reference interval for the LC-MS/MS assay was determined using sera collected in red top tubes from 97 carefully selected healthy volunteers (51 females, 46 males ages 18–65 years, employees and nonemployees of Quest Diagnostics). The following inclusion criteria were used: apparently healthy, ambulatory, community dwelling, nonmedicated adults. The exclusion criteria were as follows: any endocrine disorders, fasting glucose >100 mg/dL (>5.55 mmol/L), and abnormally high free insulin (>20 μU/mL, or 120 pmol/L) detected by the current ICMA platform.

SAMPLE TYPE AND STABILITY

Six types of collection tubes (plain red top serum, serum separator tubes, EDTA plasma, sodium heparin plasma, lithium heparin plasma, and sodium citrate plasma) were assessed using samples drawn from 10 individuals and analyzed in the LC-MS/MS assay. Sample stability was evaluated in serum over time at the following
temperature ranges: ultralow frozen (−60.0 to −90.0 °C), frozen (−10.0 to −30.0 °C), refrigerated (2.0–8.0 °C), and room temperature (18.0–26.0 °C). Freeze–thaw stability was evaluated using 10 individual patient serum samples. An initial sample with no freezing (cycle 0) was collected and 5 additional aliquots were subjected to repeated freeze–thaw cycles (1–5). Sample stability was completed by assessing the mean difference between the baseline value and the time/temperature sample value within an acceptable range of 80%–120%.

Results

EFFECTIVENESS OF IS

The labeled B chain of insulin was initially favored for the assay because it is chemically a match for the endogenous B chain. The labeled peptide was spiked into all extracted samples, QC, and calibrators as described in the Materials and Methods before analysis on the LC-MS/MS system and was used to normalize the quantitative MS results. However, to correct for the possibility of incomplete reduction of insulin, we also used intact bovine insulin as a surrogate IS added at the beginning of sample preparation to account for any procedural losses during the extraction and reduction processes. Fig. 1 shows the SRM chromatograms corresponding to coeluting bovine, endogenous human, and isotopically labeled human insulin B chain, respectively. Both ISs exhibited consistency in signal response over time, and Deming regression of 51 patients suggested equal suitability (see online Supplemental Fig. 4). We ultimately chose bovine insulin as the default IS.
because it has superior peak intensity and cost in addition to correcting for the possibility of incomplete reduction. The benefit of using an IS is demonstrated in online Supplemental Fig. 5. The correlation of LC-MS/MS results with the ICMA assay results by Deming regression was observed to dramatically improve with slope, decreasing from 1.36 (no IS) to 1.07 (bovine IS).

ASSAY PERFORMANCE
Analytical performance specifications are summarized in Table 1. The assay demonstrates a linear relationship within the reportable range of 5 to 300 μIU/mL (30–1800 pmol/L), with $R^2$ of 0.9989. We determined the insulin LOQ by assaying 6 different samples at concentrations close to the expected LOQ [1.25, 2.5, 5, 10, 15, and 25 μIU/mL (1 μIU/mL = 6 pmol/L)] and then evaluating the intraassay reproducibility in 7 runs. The lowest concentration that yielded acceptable performance was 3 μIU/mL (18 pmol/L), for which the 95% CI for the CV remained below 20%. A blank was measured 14 times and the resulting area ratios were back-calculated to establish an LOD (4 SDs from the zero concentration) of 1.8 μIU/mL (10.8 pmol/L) for insulin and a limit of blank (2 SDs from the zero concentration) of 1.4 μIU/mL (8.4 pmol/L) for insulin in stripped serum. Confirmation of insulin B-chain identity was established as described in the Methods. Mass transition ratios outside the stated tolerance limits were found in <3% of patient samples. No detectable carryover was found for concentrations below 500 μIU/mL (3000 pmol/L). Intraassay precision and accuracy for QC were generated by assaying 8 replicates. The CVs ranged from 3.0% to 7.9%, with the intraassay accuracy ranging from 80%–92%. The interassay variation over 5 days ranged from 7.1% to 14.0% and the interassay accuracy range from 87% to 96%. Assay performance was also assessed by spike and recovery experiments in patient serum and was found to have an overall recovery of 94%–113% across the measured range (Table 1).

METHOD COMPARISON
To benchmark the assay, we completed an analysis of correlations between the LC-MS/MS assay and an FDA-approved ICMA assay using 89 deidentified patient discards across the insulin concentration range of 3–180 μIU/mL (18–1080 pmol/L). Deming regression from this study revealed good agreement between the new LC-MS/MS method with the ICMA assay ($y = 1.15x - 0.89$) (Fig. 2).

REFERENCE INTERVAL DETERMINATION
In this investigation, we used samples from 97 carefully selected healthy donors to complete a reference interval study (Fig. 3). The data were found to be nongaussian. The 95th percentile data were used to establish a reference interval of <13.7 μIU/mL (82.2 pmol/L).

ASSAY QC
We used QC procedures according to Westgard rules within the assay to allow determination of passing vs failing runs as well as to identify biases within the data attributable to analytical issues. QC controls were evaluated from 2 different sources. The first were Bio-Rad Immunoassay/TDM controls, QC serum samples with known concentrations of insulin. The second type of QC sample was a low insulin concentration in-house serum pool that we prepared by determining the insulin concentration after combining the serum from a

<table>
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<th>Table 1. Performance of the LC-MS/MS assay for insulin.</th>
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<td><strong>Sensitivity</strong></td>
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<td>Insulin in stripped serum, μIU/mL (pmol/L)</td>
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* LOB, limit of blank.
large population of donors. Ranges for the in-house QC serum and Bio-Rad controls were determined for each new lot. Working ranges for the QCs were established by measurement of some 50–70 observations per level and in multiple replicates over 5 days. The results were statistically analyzed to obtain mean values (16.1, 46.5, and 189.5 μIU/mL or 96.6, 279, and 1137 pmol/L) and CVs for each QC level (17.4%, 12.4%, and 8.2%, respectively). These data were then used to set the 2- and 3-SD limits for QC acceptability.

During sample analyses, all QC levels were tested at the beginning of each assay with multiple replicates of the QCs interspersed throughout the assay thereby bracketing patient samples.

**SAMPLE TYPE AND STABILITY**

The insulin assay was validated for human serum collected in plain red-top tubes. A comparison of insulin concentrations determined in blood collected in different types of tubes revealed that sodium and lithium...
heparin gave equivalent results and therefore are acceptable sample types (data not shown). Sample storage conditions were evaluated in terms of reproducibility in quantification over time with acceptable measurements falling within $\pm 20\%$ of day 0 (see online Supplemental Fig. 6). Insulin in patient serum was stable for at least 86 days at $-80\,^\circ C$. However, at $-20\,^\circ C$ it was observed that measured insulin concentrations in a portion of serum samples dropped after samples were stored for 35 days. Insulin was stable in serum for only 1 day at room temperature. Furthermore, insulin concentrations in some samples appeared to decline on the second day of storage at refrigerated temperatures, as indicated by the increased SD, whereas samples from other patients showed exemplary stability. After analyzing all the stability results it was determined that samples for the LC-MS/MS insulin assay should be stored and shipped frozen at $-20\,^\circ C$ or lower for the short term (less than 1 month). Longer-term storage should be at $80\,^\circ C$ or lower. Finally, the data indicate that insulin concentrations were unchanged by 5 freeze–thaw cycles (see online Supplemental Fig. 6).

**INTERFERENCE STUDIES**

We performed interference studies by examining stripped sera from different sources as well as testing over 100 patient samples to look for nonspecific interferences. The effects of varying degrees of hemolysis, lipemia, and bilirubin (mild, moderate, or gross) on the accuracy of insulin measurements were also investigated. The effects of hemolysis/lipemia/bilirubin on insulin determinations were evaluated by spiking different concentrations of insulin in patient samples with low, medium, and high hemolysis/lipemia/bilirubin. An acceptance level of $80\%-120\%$ recovery was used to assess whether the assay was adversely impacted by interference. No dependence between insulin concentrations and the level of lipemia or concentration of bilirubin was observed. In contrast, there were unacceptable recoveries of insulin from grossly hemolyzed sera. Thus, hemolysis constituted a criterion for sample rejection (data not shown).

**Discussion**

The application of HPLC interfaced with MS for protein and peptide analysis in the clinical laboratory environment has always been very challenging compared to the current antibody-based automated immunoassays. Insulin proved to be no exception to this observation. First, any new assay is required to provide excellent selectivity and specificity regardless of the complexity of the human serum. Second, the usual concentration range for insulin in human blood is quite low; some immunoassays have lower LOQs as low as 2 $\mu$IU/mL, equivalent to 1.8 fmol of insulin in the 150 $\mu$L of serum we use for our assay. Third, any assay that is going to be routinely used in a clinical laboratory must be simple and highly reproducible as well as being robust and able to accommodate high sample throughput. Each LC run takes $<8$ min, and by multiplexing the assay, data can be obtained for each sample in 2 min. On average, a 96-well plate takes $<4$ h to run. Sample preparation throughput is determined by extraction and reduction incubation steps (about 2 h). Thus, we have developed an LC-MS/MS method which meets all the criteria for routine use in a clinical laboratory.

A unique feature of the LC-MS/MS insulin assay reported here is the consistency and harmonization of the NIBSC insulin standard and the synthesized recombinant human insulin. Reference to the gold standard WHO reference reagent purchased directly from NIBSC with a designated biological activity of 3 IU per vial should help to avoid the standardization issues that have plagued the currently available antibody-based immunoassays, which each use their own working standards developed by the manufacturers. The assay has been validated, meeting internal policy in compliance with CLIA 88 493.1253 guidance, with an LOD of 1.8 $\mu$IU/mL (10.8 pmol/L) and lower LOQ of 3 $\mu$IU/mL (18.0 pmol/L). To confirm the applicability of this assay, a correlation between the LC-MS/MS assay and an FDA-approved ICMA was completed, and the resulting Deming regression revealed good agreement between both methods in the lower concentration range. A new reference interval has also been established for the LC-MS/MS assay.
The methodology for this assay involves reduction of intact insulin and measurement of the free B chain. This approach provides valuable information for distinguishing the endogenous insulin from therapeutic insulin analogs. All the commercial synthetic forms of insulin have alterations affecting the C-terminal amino acids on the B chain. The SRM mass filters used provide molecular structural details confirming the authenticity of the insulin B chain signal being measured, while ignoring the synthetic analogs which are routinely detected as insulin with the use of immunological methods (unpublished). In addition, the requirement that proinsulin not be recognized by the immunoassays is also fulfilled by the selectivity of the SRM-based quantification method (19, 20).

Conclusion

A simple, high-throughput, quantitative LC-MS/MS insulin assay traceable to the NIBSC standard has been successfully developed and validated to CLIA guidelines. The method combines molecular specificity with quantitative performance and standardized analysis and provides reference intervals, making this approach a candidate to be considered as a reference method for insulin testing (19, 20).

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