Standardization of Insulin Immunoassays: Report of the American Diabetes Association’s Workgroup

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Supplemental Information: Complete Materials and Methods

Background

The ADA Workgroup agreed on two major points: 1) the standardization effort would primarily target commercially available assays that were verified to measure insulin with negligible cross-reactivity with proinsulin and proinsulin cleaved intermediate products, and 2) insulin concentrations be reported in Système International (SI) units, pmol/L, rather than the traditional units based on insulin biologic activity per mg of insulin standard preparation. Highly purified, biosynthetic/semi-synthetic human insulin was used as the basis for accurate SI molar units of insulin, assuming a molecular weight of monomeric insulin of 5,808 Da. A multiplication factor of 6.0 was used to convert concentrations in µU/L to pmol/L (1,2).
**Study Design**

The major suppliers of assays that claimed to be insulin specific were invited to participate. The following manufacturers participated in at least one of the two phases of the investigation: Abbott Japan; Bayer HealthCare, USA; Beckman Coulter Inc., USA; Dako Cytomation, Ltd., United Kingdom; Diagnostic Products Corporation, USA; Diagnostic Systems Laboratories, USA; Linco Research Inc., USA; Mercodia, Sweden; and Roche, Germany. An assay from Tosoh Bioscience Inc., Japan, was evaluated in-house at the NWRL.

Immunoassays evaluated in at least one of the two phases included, 7 noncompetitive chemiluminescence assays on dedicated instrumentation, 2 competitive radioimmunoassays (RIA), and 3 noncompetitive enzyme-linked immunoassays (ELISA). Two manufacturers used more than 1 assay. The participating manufacturers requested their respective performance remain anonymous; consequently, each assay was assigned an arbitrary number for reporting results.

For the first phase of the protocol we evaluated: 1) the comparability of insulin concentrations obtained by the different assays using each assay manufacturer’s product calibrators, 2) the possibility to harmonize results by calibrating the different assays using a highly purified recombinant insulin preparation as a proposed reference material for insulin (PRMI), and 3) analytical imprecision. Duplicate analyses were performed on three separate days on samples from 40 individual donors representing a wide range of
insulin concentrations. The protocol was performed twice for each assay, first with the assay calibrated using the manufacturer’s product calibrators, and second using the PRMI as a calibrator diluted in the assay’s recommended diluent.

For phase 2 of the study we aimed to assess: 1) the insulin recovery of the different assays, and 2) the specificity of each assay by determining the degree of cross-reactivity of intact human proinsulin (hPI) and the proinsulin intermediate metabolites, split (32,33) hPI and des (64, 65) hPI. Replicate analyses were performed on a set of samples prepared by spiking a serum pool obtained from donors with very low insulin concentration with different amounts of purified insulin, proinsulin and proinsulin intermediates, as described later. In addition, each participant prepared dilutions of PRMI using the assay’s recommended diluent and assayed each dilution to evaluate parallelism of insulin recovery to the recovery from insulin added to a serum pool.

**Preparation of Human Insulin Standard**

LINCO AB-P Insulin Assay buffer (LINCO Research, Inc., St. Charles, MO) (containing phosphate, EDTA, NaCl, RIA-grade bovine serum albumin and Na azide) was selected for use to prepare the PRMI stock solution of insulin because of its simple composition, potential good compatibility with commercial insulin immunoassays and the known stability of insulin in this matrix. Although no formalized stability assessment was performed in conjunction with this study, prior experience with this buffer indicates that insulin shows excellent long-term stability under frozen (−20 °C and −70 °C) conditions and for at least 72 hours at either ambient temperature or 2–8 °C. Furthermore insulin
stored in AB-P Insulin Assay Buffer is stable through at least 4 cycles of freezing and thawing (Ronald Bowsher, unpublished results). Highly-purified reference human insulin preparations were kindly provided by Novo Nordisk (Denmark) and Eli Lilly and Company (Indianapolis, IN, USA), with a potency of 6.0 nmol/IU or 28.7 IU/mg pure insulin (1,2). The working stock solution of PRMI (at a concentration of 250 nmol/L) was prepared at LINCO Diagnostic services, Inc. (St. Charles, MO, USA) by hydrating the lyophilized peptide with 0.01 mol/L HCl, followed by dilution in AB-P Insulin Assay buffer. Aliquots were pipetted and stored frozen in polypropylene vials at −70 ºC. After verification of the ‘nominal’ insulin concentration by a validated ‘in-house’ RIA (LINCO Diagnostic Services, St. Charles, MO), the frozen vials were shipped on dry-ice to the NWRL at the University of Washington for distribution to the phase 1 participants. Results from the RIA were used only to confirm the nominal concentration and not for an assignment of insulin concentration.

The PRMI was shipped to manufacturers on solid CO₂. Participants were instructed to thaw the vial of PRMI at ambient temperature, mix thoroughly, separate the material into 50- to 100-µL aliquots, and to store the vials at −70 ºC for subsequent use. A new vial of PRMI was thawed on each day of use. One manufacturer did not store the PRMI from phase 1 and was sent another vial to perform phase 2 analysis.

**Preparation of Masked Plasma Samples**

To obtain the requested large range of insulin values needed for phase 1, blood was collected from fasting individuals (N=32) or from individuals in postprandial state (N=8).
Of importance none of the donors has received exogenous insulin. After obtaining informed consent, blood was collected by venipuncture in EDTA-containing Vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ); after 30 min on ice, samples were centrifuged at 1,500 g for 15 min in a refrigerated centrifuge, with plasma from each donor separated and pooled in a 50-mL conical tube. Samples were then dispensed into appropriately labeled 2- mL cryo-vials (1 mL of plasma/vial), flash frozen in a liquid nitrogen chamber, and stored at −70 °C until analyses were performed locally or samples shipped on solid CO₂ to the participating manufacturers. The elapsed time from collection of blood to freezing of aliquots was less than 2 hours. Analyses of each sample to estimate insulin concentrations were performed at the NWRL by RIA (HI-14K, Linco Research, Inc, St. Charles MO).

For phase 2, whole blood was collected from 7 fasting donors who had very low concentrations of insulin into tubes containing no anti-coagulant. Samples were allowed to clot at room temperature for 30 min, followed by centrifugation at 1,500 g for 20 min in a refrigerated centrifuge. After processing, sera were pooled, mixed, and distributed into 7 sterile flasks in a volume of 60 mL per flask using a volumetric pipette, and an additional 250 mL were transferred into a sterile bottle. The flasks and bottle were kept on ice during handling.

**Supplementation with PRMI**

Nothing was added to the first flask. Different amounts of PRMI were added to each of the other flasks, using a calibrated pipette, to achieve a final concentration of added
insulin of 60, 120, 240, 360, 480, and 600 pmol/L of serum, respectively. Unspiked and spiked serum samples were then transferred into appropriately labeled cryovials (1 mL of serum/vial), flash-frozen in a liquid nitrogen chamber, and then stored at −70 ºC until analyzed or shipped on dry ice to the participating manufacturers. The elapsed time from collection of blood to the freezing of aliquots was less than 2 hours.

**Supplementation with Human Proinsulin and Cleaved Proinsulin Metabolites**

To evaluate the specificity of the insulin assays, human serum was supplemented with high concentrations of either intact proinsulin or one of its cleaved junctional metabolites. Intact human proinsulin (hPI), (lot RS0314) and its cleaved metabolites, des (64,65) hPI (lot A18-4U6-239-1) and split (32,33) hPI (lot A18-3U1-70A-1), were graciously provided by Eli Lilly and Company. Prior to adding the hPI or metabolites, PRMI was added to the 250-mL serum bottle to yield a final concentration of 40 μU of added insulin/mL to ensure that all assay values would be measurable. This preparation was then shipped on frozen CO₂ by overnight service to LINCO Diagnostic Services, Inc., where the 250-mL serum pool was thawed and promptly divided into 5 equal-volume aliquots of 50 mL each. Four of the 5 serum aliquots were then supplemented with 1 mmol/L Pefabloc SC (AEBSF, Roche), an irreversible serine protease inhibitor, to stabilize hPI and its metabolites. One serum aliquot was not supplemented with Pefabloc SC, and served as a control to assess the effect of the Pefabloc SC addition on the performance of the various insulin immunoassays. The other 3 aliquots were supplemented with one each of the respective proinsulin peptides. The various standard solutions were prepared by adding an appropriate volume of 0.01 mol/L HCl or
phosphate-buffered saline (PBS) to each vial and reconstituting the pre-weighed lyophilized peptide by gentle swirling. After reconstitution, an appropriate volume of each peptide solution was added to the sera aliquots containing Pefabloc SC to yield 6,000 pmol/L intact hPI, 6,000 pmol/L of hPI split (32,33) and 600 pmol/L of des (64,65) hPI, respectively. In each case the total volume of the peptide solution added was less than 0.5% of the serum volume.

Statistical Analysis

An analysis of variance (ANOVA) procedure was performed on the replicate results for samples from 40 patients to estimate the among-assay variance measured using each respective manufacturer’s product calibrators and using PRMI as a common calibrator for each assay. The assay was treated as a random factor in a three-factor model with patient sample ID and day of analysis acting as random blocking factors. An ANOVA model was also used to test for a difference between the among-assay variance obtained for results based on manufacturer’s product calibrators and for results based on using PRMI as a common calibrator. In this model, the calibrator (manufacturer’s product calibrator versus PRMI common calibrator) was treated as a fixed factor with assay, patient sample ID, and day of analysis acting as random blocking factors.

An ANOVA procedure was performed for samples from each of 2 groups of 20 patients to estimate the within-assay variance using the results for each assay calibrated with its manufacturer’s product calibrator. The 2 groups corresponded to concentrations below and above approximately 69 pmol/L. Six replicates of each sample’s results were
available, except for assays 6 and 9, which only provided 3 replicates for each sample. Separately for each assay and each of the 2 groups, a two-factor model, with participant sample identification and day of analysis acting as random factors, was used to generate an estimate of the within-assay variance: computed by adding estimates of the among-day variance and the within-day (run) variance obtained from the ANOVA as an estimate of the variance associated with a single measurement.

For recovery of insulin added to the serum pool, the mean of 12 replicates was used for each insulin measurement. The insulin in the base serum pool was subtracted from the insulin in each addition sample to obtain the insulin recovered. The insulin recovered was expressed as a fraction of the insulin added to prepare each sample. To average the recovery over the concentration range, the fraction of insulin recovered at the minimum and maximum added concentrations was calculated from the least squares regression of the actual fraction of insulin recovered onto the amount of insulin added at each of the concentrations between 120 and 600 pmol/L.

For recovery of insulin from dilutions with each manufacturer’s recommended diluent, the mean of 12 replicates was used for each insulin measurement. The recovery of insulin from dilutions of PRMI was compared to the recovery of insulin from standard additions of PRMI to a serum pool by least-squares linear regression of the recovered onto the expected concentrations for each condition. Slopes were calculated, standard errors of the slopes were computed, and a two-sided t-test was performed to test the null hypothesis that there was no difference between the slopes.