Standardization of C-Peptide Measurements

Randie R. Little,1* Curt L. Rohlfing,1 Alethea L. Tennill,1 Richard W. Madsen,2 Kenneth S. Polonsky,3 Gary L. Myers,4 Carla J. Greenbaum,5 Jerry P. Palmer,6 Eduard Rogatsky,7 and Daniel T. Stein7

BACKGROUND: C-peptide is a marker of insulin secretion in diabetic patients. We assessed within- and between-laboratory imprecision of C-peptide assays and determined whether serum calibrators with values assigned by mass spectrometry could be used to harmonize C-peptide results.

METHODS: We sent 40 different serum samples to 15 laboratories, which used 9 different routine C-peptide assay methods. We also sent matched plasma samples to another laboratory for C-peptide analysis with a reference mass spectrometry method. Each laboratory analyzed 8 of these samples in duplicate on each of 4 days to evaluate within- and between-day imprecision. The same 8 samples were also used to normalize the results for the remaining samples to the mass spectrometry reference method.

RESULTS: Within- and between-run CVs ranged from <2% to >10% and from <2% to >18%, respectively. Normalizing the results with serum samples significantly improved the comparability among laboratories and methods. After normalization, the differences among laboratories in mean response were no longer statistically significant (P = 0.24), with least-squares means of 0.93–1.02.

CONCLUSIONS: C-peptide results generated by different methods and laboratories do not always agree, especially at higher C-peptide concentrations. Within-laboratory imprecision also varied, with some methods giving much more consistent results than others. These data show that calibrating C-peptide measurement to a reference method can increase comparability between laboratories.

© 2008 American Association for Clinical Chemistry

1 Department of Pathology & Anatomical Sciences, University of Missouri School of Medicine, Columbia, MO; 2 Department of Statistics, University of Missouri School of Medicine, Columbia, MO; 3 Department of Medicine, Washington University School of Medicine, St. Louis, MO; 4 Centers for Disease Control and Prevention, Division of Environmental Health Laboratory Sciences, Centers for Environmental Health, Chamblee, GA; 5 Berunaya Research Institute, Seattle, WA; 6 University of Washington and VA Medical Center, Seattle, WA; 7 Department of Medicine and GCRC Analytical Core Laboratory, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY.

* Address correspondence to this author at: Diabetes Diagnostic Laboratory, M767, Department of Pathology & Anatomical Sciences, University of Missouri School of Medicine, 1 Hospital Dr., Columbia, MO 65212. Fax 573-884-8823; e-mail LittleR@health.missouri.edu.

Received November 28, 2007; accepted March 5, 2008.
Previously published online at DOI: 10.1373/clinchem.2007.101287

Nonstandard abbreviation: LC-MS, liquid chromatography–mass spectrometry.
Materials and Methods

This study was conducted by the Diabetes Diagnostic Laboratory at the University of Missouri–Columbia with input from the C-Peptide Standardization Committee (see Appendix in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue6). The University of Missouri Health Sciences Institutional Review Board approved the protocol, and all participants gave written informed consent. Forty samples were collected from 24 healthy nondiabetic volunteers after they had fasted overnight and/or 60 min after they had consumed a standard meal (Boost™; Novartis Medical Nutrition). Venous blood was collected into serum tubes without additives and allowed to clot at room temperature for 30 min before centrifugation. Serum aliquots were removed from each sample, shipped frozen on dry ice, and kept at −70 °C in the respective laboratories before analysis (within 6 months of sample collection). A single-use frozen aliquot was provided for each analysis on each day. The laboratories (see Appendix in the online Data Supplement) were instructed to analyze specific samples on 4 different days in the same manner as they would analyze clinical samples and to provide a single result for each sample. Because EDTA-treated plasma containing aprotinin was the only sample type that had been validated for the LC-MS reference method, we also collected a small amount of EDTA-anticoagulated blood from each study participant at each time point for LC-MS analysis, centrifuged the samples, and treated the plasma with aprotinin (50 mL/L plasma).

Serum samples were sent to 15 laboratories in 5 countries. Because one of the laboratories used 3 different C-peptide assay methods, we evaluated a total of 17 laboratory/method combinations. The Millipore/Linco RIA, the Siemens/DPC Immulite 2000 chemiluminescence immunoassay system, and the Siemens/DPC RIA kit were used by 3 laboratories each. The Tosoh Bioscience AIA-600II immunoassay and the Fujirebio Lumipulse chemiluminescent enzyme immunoassay were used in 2 laboratories each. The other methods (the Adaltis RIA, the Dako ELISA, the PerkinElmer time-resolved fluoroimmunoassay, and an in-house immunochemiluminescent assay) were used by 1 laboratory each. The EDTA-containing plasma aliquots were shipped to the Albert Einstein College of Medicine of Yeshiva University for analysis by isotope-dilution 2-dimensional LC-MS, which has been shown to have a between-run CV of <2% (11).

Eight samples from different individuals were used as calibrators, and the samples were chosen from preliminary C-peptide measurements to cover a wide concentration range (final LC-MS concentration range, 0.43–1.81 nmol/L). Each of the 15 laboratories analyzed each of the 8 samples in duplicate (masked duplicates) on each of 4 days to provide estimates of within- and between-day imprecision. The same 8 samples were also used to normalize the results from the remaining 32 samples. The LC-MS results for the corresponding plasma samples were used as the assigned value for each of these serum-sample calibrators.

Data were analyzed with Microsoft Excel and SAS (SAS Institute) software. All C-peptide results were converted to SI units (1 µg/L = 0.331 nmol/L) before data analyses. For each laboratory/method combination we assumed a constant CV within days and between days. The within-run CV was calculated for each sample pair for each laboratory/method combination. The mean CV for each laboratory/method was then calculated to estimate the within-run imprecision for each laboratory.

Thirteen of the 18 laboratories provided duplicate results for each of the 8 calibrator samples for each of 4 days (duplicate results × 4 days = 8 observations per laboratory/method per calibrator); however, because we had blinded the samples and because a few of the methods have a very limited linear range, we had to reanalyze diluted samples in a few cases when the results were outside of an assay’s linear range. Therefore, to estimate the between-run CV for each laboratory, we adopted a simulation approach. For each of the 8 calibrator samples, we randomly chose 1 observation from each day and calculated a CV. We then repeated this procedure 100 times and calculated a mean for the set of 100 sample CVs determined for each sample for each laboratory/method. To test for statistically significant differences among laboratories, we first log-transformed the mean values to approximate a normal distribution and then used a 1-way ANOVA to test for the equality of means (General Linear Models procedure in SAS). The results of Levene’s test of homogeneity of variances indicated unequal variances in the values across laboratories, so we used Welch’s method for the analysis (13, 14).

To look for significant differences in the mean laboratory response, we used the 32 noncalibrator samples to assess the differences between laboratory/method combinations. We assumed that the residual variances differed among the different laboratory/methods and hence fit a heterogeneous variance model. This analysis was carried out with the Mixed Procedure in SAS.

The 8 sample calibrators were used in each laboratory to calibrate each day’s run to the LC-MS reference method. Because scatter plots of the data indicated that the imprecision in response increased with increasing C-peptide concentration, the model assumed a constant CV (i.e., an increasing SD). We used weighted regression methods with the weights inversely propor-
tional to the variance. We performed iterative re-weighting because the variance had to be estimated from the modeled regression results. For each laboratory/method combination, we fitted second-degree equations to data consisting of laboratory/method values for the serum standards plotted on the y-axis and the “true” values for the standards on the x-axis, and we used weights based on the assumption of a constant CV in the weighted regression analyses. Because the coefficient for the squared term was not significantly different from zero in all cases, we performed the normalization by using simple linear relationships. Using the normalized results for the 32 samples, we then repeated the analysis described in the previous paragraph to look for significant differences in the mean laboratory response.

Results

Fig. 1 shows the within-day vs between-day CV estimates for each laboratory/method. The laboratory/method combinations with the lowest within- and between-laboratory imprecision (CV <4% for both) included the Fujirebio Lumipulse chemiluminescent enzyme immunoassay, the PerkinElmer time-resolved fluoroimmunoassay, and the Tosoh AIA-600II immunoassay. The Adalitis RIA and one of the DPC RIA laboratory/methods showed the greatest imprecision, with between-run CV values of 15%. All other laboratory/method combinations had between-run CVs between 5% and 11%.

Fig. 2 summarizes the C-peptide results before (Fig. 2A) and after (Fig. 2B) normalization with the 8 serum calibrators. Before normalization, there were significant differences between laboratory means (P < 0.0001). The least-squares means ranged from 1.55–1.95. The LC-MS results were lower than for all of the other methods except for 2 samples (nos. 14 and 30, both postprandial samples). The fasting samples (nos. 6 and 19) from the same 2 donors did not show the same relationship, and proinsulin concentrations in these outlier samples were not higher than those in many of the other samples. After normalization, there were no significant differences in the mean responses (P = 0.24). The least-squares means ranged only from 0.93–1.02. Because the values were normalized to the mass spectrometry reference laboratory results for each laboratory, it is not surprising that the means of the transformed values were all close to the mean value (0.99 nmol/L) obtained by the reference laboratory.

Discussion

We have shown that normalization of C-peptide results for patient samples that have been assigned values by a reference method greatly reduces the imprecision among methods and laboratories. It is important to note that there are large differences in within-day and between-day imprecision among the available C-peptide assay methods. Normalization of the results to a reference method may not necessarily achieve consistent results for methods that demonstrate high imprecision.

The LC-MS method used in the present study has been validated only with EDTA-treated plasma samples containing aprotinin. Because the presence of
EDTA in a sample is not acceptable for some routine methods, the LC-MS results for EDTA-containing plasma samples were transferred to the serum samples used by the other laboratories. In preliminary studies with LC-MS, we compared serum samples with matched EDTA-treated plasma samples containing aprotinin that had been stored at $\text{70 } ^\circ\text{C}$ for 2–30 days. The mean values of the serum and plasma samples were comparable throughout the study. Studies are currently underway with a larger group of samples to better define the relationship between serum samples and preserved plasma samples and to validate the use of the LC-MS method with serum samples.

Until a more generalized standardization program is implemented, the comparability of C-peptide results between laboratories must be addressed before the initiation of large-scale trials involving multiple laboratories performing C-peptide analyses.

**Grant/Funding Support:** This project was funded by the Centers for Disease Control and Prevention through a funding contract (No. 200200409985) supported by the National Institutes of Diabetes and Digestive and Kidney Diseases Special Statutory Funding Program for Type 1 Diabetes Research.

**Financial Disclosures:** None declared.

**Acknowledgments:** We thank Judith Fradkin and Lisa Spain for their support. We also thank Donghua Huang of the Diabetes Diagnostic Laboratory, University of Missouri, for her technical assistance with this project.

**References**


**Fig. 2.** Comparison of C-peptide results among laboratory/method combinations. Results are shown before (A) and after (B) normalization with serum calibrators. Samples are presented in the order of C-peptide concentration, which is based on the mean of all routine methods. Each symbol represents a different method. CLEIA, chemiluminescent enzyme immunoassay; FIA, fluoroimmunoassay.