An Improved Radioimmunoassay of C-Peptide and Its Application in a Multiyear Study

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A commercial radioimmunoassay (RIA) for human proinsulin C-peptide was modified to improve its ruggedness and specificity, to decrease the influence of specimen matrix, and to shorten "hands-on" time. In the new protocol, we prepare calibrators in a C-peptide-free serum pool, prepared by treatment with activated charcoal (biological matrix), instead of in a defined matrix. This yielded essentially 100% analytical recoveries for C-peptide concentrations up to 300 pmol/L, a broader analytical range. We also corrected calibrators and unknown samples for nonspecific binding (NSB). Decreasing the concentration of ethanol (from 950 to 880 mL/L) for differential precipitation of the antigen–antibody complex resulted in an NSB of <10%, while maintaining high bound/total count percentages for samples and calibrators. C-peptide is thermally unstable without aprotinin at -20°C and with or without aprotinin at 4°C or above, but multiple freeze-thaw cycles do not affect C-peptide in serum. The modified C-peptide assay was applied to plasma from a multiyear study (fasting and post-carbohydrate-challenge subjects). During the four years of the study CVs ranged from 1.9% to 8.6% for replicate analyses of C-peptide in samples with concentrations ≤500 pmol/L. Between-run CVs were 3.8% to 8.2%, total CVs 3.8% to 10.7%.

Additional Keyphrases: quality control, variation, source of

Measuring the connecting peptide (C-peptide) in proinsulin provides an assessment of beta-cell secretory activity, indicating the extent to which the person with diabetes mellitus retains the capacity to secrete insulin. C-peptide and insulin are secreted in a 1:1 molar ratio, but the half-life for C-peptide is much longer than that of insulin (1), leaving more C-peptide available in the circulation for quantification. Measurement of C-peptide is preferred over that of insulin in insulin-requiring diabetics because of interference in insulin assays by exogenous insulin and circulating antibodies to insulin, which are often found in persons on insulin therapy (2–4). Baseline (fasting) and post-carbohydrate-challenge measurements in plasma are more useful than measurements made after fasting alone (5).

As the central laboratory for the Early Treatment Diabetic Retinopathy Study (ETDRS) (6), we evaluated and refined currently available analytical methods for analyzing C-peptide. The ETDRS is a randomized clinical trial sponsored by the National Eye Institute to evaluate the efficacy of several treatment strategies involving photocoagulation to manage patients with nonproliferative and early proliferative diabetic retinopathy. The study is also evaluating the effect of acetylsalicylic acid on the course of diabetic retinopathy and other vascular sequelae of diabetes. From April 1980 through August 1985 the ETDRS research group enrolled 3711 persons with diabetes who had various manifestations of diabetic retinopathy. Preliminary results of this ongoing clinical trial (7) show that focal photocoagulation is effective in treating clinically significant macular edema.

Three of the 22 ETDRS Clinical Centers participated in an ancillary research project and collected baseline specimens from 582 study patients for C-peptide measurements, to provide an objective measure of beta-cell secretory function. These data were compared with ETDRS clinical criteria for classifying diabetes (8).

Here we present studies of the modifications of a commercial C-peptide method that we made to minimize nonspecific binding and tracer precipitation, to improve analytical precision, to establish quality-control parameters, to improve analytical efficiency, and to establish safe conditions for sample shipment and storage. We describe our experience with the consistent performance of this assay during four years of continual use.

Materials and Methods

Apparatus

All radioactivity measurements were done with an Ultragamma (Model 1280) or a Rackgamma II (Model 1270) gamma counter (LKB-Wallac, Turku, Finland).

Reagents

Human C-peptide radioimmunoassay kits were purchased from the NOVO Research Institute, Bagsvaerd, Denmark. This kit supplied synthetic human C-peptide (31 amino acids) for preparing calibrators, 125I-labeled Tyr-C-peptide (~210 Ci/g), and anti-human C-peptide antiserum raised in guinea pigs (lot no. M1230) (9). Each kit contained materials sufficient for ~1500 assay tubes when used according to the supplied protocol described by Heding (10).

Aprotinin (10 000 kallikrein inhibitor units (KIU)/mL), a proteolytic enzyme inhibitor, was purchased as "Trasylol" (Mobay Chemical Corp., FBA Pharmaceuticals, New York, NY 10022) in sterile glass ampules and was transferred to sterile vials with resealable septa for convenient use. Human sera for the preparation of pools were obtained from the Centers for Disease Control serum bank from nondiabetic volunteers. Common reagent chemicals were obtained as ACS grade from Fisher Chemical Co., Norcross, GA 30091, or Sigma Chemical Co., St. Louis, MO 63178.

Patients' Samples

Samples were obtained for ETDRS from three clinical centers: the Joslin Diabetes Center, the University of Minnesota, and the University of Wisconsin. Two sets of diabetes classification definitions were developed in the ETDRS (8). According to the "strict" set of definitions, ETDRS patients were classified into three groups: Strict Type I (44.6%), Strict Type II (19.8%), and another intermediate group
were refrigerated (47721), II dependent (35.6%). With a "broad" set of definitions, all types of patients were categorized into one of two groups: Broad Type I (57.1%) and Broad Type II (42.9%). As outlined by the National Diabetes Data Group (11), Type I refers to insulin-dependent diabetes mellitus and Type II refers to non-insulin-dependent diabetes mellitus. The intermediate group in the "strict" set of definitions includes patients who could not be categorized into the Strict Type I or Strict Type II groups.

Patients were required to fast from midnight until the morning of the clinic visit. Upon arrival, a blood specimen was drawn, after which the patient drank one 12-oz. (~336 mL) can of chilled "Sustacal" (Mead-Johnson, Evansville, IN 47721), producing a 50-g carbohydrate load; a "stress" blood sample was drawn 1.5 h later. For all blood specimens, an arm tourniquet was applied and blood was drawn from an antecubital vein and collected in a lavender-top, EDTA-containing evacuated tube. Maintaining sterile technique, the clinic technologist injected 0.2 mL of aprotinin into the evacuated tube immediately after blood collection to inhibit enzymatic degradation of C-peptide (9, 12, 13). The tube was refrigerated until centrifuged, and 1 mL of plasma was shipped on solid CO₂ and subsequently stored at −70 °C until the day of the C-peptide analysis.

Prepared Solutions

"FAM". According to the NOVO kit method, FAM was prepared to give final concentrations of 0.04 mol/L phosphate buffer, pH 7.4, containing 1 g of human serum albumin and 0.24 g of human thimerosal per liter.

NaFAM. To the FAM solution we added NaCl and human serum albumin to give concentrations of 6 g/L and 60 g/L, respectively. This was used to dilute antisera and samples according to the NOVO method.

Ethanol–FAM. We combined 960 mL of ethanol (950 mL/L), 18 mL of FAM, and 162 mL of water to wash the precipitate from the separation step in accordance with the NOVO protocol.

Normal serum quality-control pool. A quality-control pool (QC5178) was prepared from normal nonobese human serum stored at −70 °C until use. Sera were thawed, combined, and mixed thoroughly. Sodium azide was added to give a final concentration of 2 g/L. The serum pool was then subjected to nitrogen-pressure filtration through a stack of filters with decreasing pore sizes from 8 to 0.22 μm. Aprotinin was added to the filtrate to give a final concentration of 500 KIU/mL of serum. We then dispensed the pool in 1-mL aliquots into 2.5-mL glass Wheaton vials, stoppered, and crimped the vials, and froze the aliquots to −20 °C initially, and then stored them at −70 °C until the day of use.

Zero C-peptide serum pool. Approximately 3000 mL of frozen human serum was thawed and treated twice with Norit neutral, activated charcoal (1/4) to remove C-peptide, as follows. Add charcoal (30 g/L) to the thawed serum pool, mix slowly at room temperature for 8 h, and then let stand overnight without mixing. Centrifuge the mixture at 8000 × g for 30 min, then filter the supernate through Whatman SP filter paper. Next add charcoal, 50 g per liter of serum, with mixing for 5 h at room temperature, centrifuge, and filter through the Whatman filter paper. Add 750 mg of dextran sulfate, sodium salt, dissolved in 10 mL of water, to the remaining 2500 mL of the filtered serum, stir for 15 min at room temperature, and then let stand for 1 h. Filter the mixture under pressure through a filter stack (pore sizes decreasing from 8 to 0.22 μm). Add sodium thimerosal and aprotinin (final concentrations 0.24 g/L and 500 KIU/mL, respectively) and sterile-filter the pool under pressure through 0.22-μm (pore-size) filters. We used the resulting pool, QC5076, as the base for preparing C-peptide calibrators and analytical recovery samples and as the diluent for samples with high concentrations of C-peptide.

Analytical-recovery pools. Two pools for analytical-recovery studies were prepared from the zero C-peptide pool (QC5078) as follows. Pool QC5078.6 was prepared by diluting 8 pmol of the NOVO C-peptide calibrator to 100 mL with QC5078 at 20 °C in a Class-A volumetric flask to yield a final C-peptide concentration of 60 pmol/L. We dispensed 0.5-mL aliquots into 1.0-mL glass Wheaton vials, capped them, and stored them at −40 °C. Similarly, pool QC5078.12 was prepared to a final concentration of 120 pmol/L. Pools QC5078.6 and QC5078.12 were also used as quality-control pools throughout the study. We additionally determined the analytical recovery of the modified method by adding specific quantities of the 1000 pmol/L calibrator from the C-peptide kit to the first incubation mixture when we were analyzing QC5078 with the RIA.

Evaluation Studies

Effect of ethanol concentration on the separation step. Ethanol concentrations from 800 to 960 mL/L were tested for the efficiency of differentially separating antibody-bound C-peptide from free C-peptide in pools QC5078.6 and QC5078.12. Counts per minute ratios of antibody-bound/total (B/T) and nonspecifically bound (NSB)/total for the QC5078.6 and QC5078.12 pools were determined at each ethanol concentration.

Comparison of the NOVO and the modified methods. Statistical differences between the NOVO kit protocol and the modified improvements were tested with the C-peptide calibrators prepared in NaFAM. The proposed changes tested included the differential precipitation of bound from free C-peptide with 880 mL/L ethanol, no pellet wash after centrifugation, and no NaOH addition to the pellet before counting, as opposed to the NOVO method, which involves precipitation with 950 mL/L ethanol, a pellet wash with ethanol-FAM, and the addition of 0.05 mol/L NaOH to the pellet.

Effect of matrix on the calibration curve. C-peptide calibrators at 0, 10, 30, 50, 100, 200, 300, 500, and 1000 pmol/L were prepared with Class-A glassware at 20 °C in a defined matrix (NaFAM) or a serum matrix (QC5078 base pool). The modified method was used with both sets of calibrators to determine the degree of matrix bias on the method.

C-peptide stability. The stability of C-peptide was tested in pooled, normal human sera, with or without aprotinin, 500 KIU/mL. Samples were aliquoted into glass vials, stoppered, and stored at −20, 4, 23, or 37 °C for eight, 15, 22, or 29 days and then were frozen to −70 °C until the day of analysis. Baseline samples were stored at −70 °C throughout the incubation times. The samples were divided into two groups—with and without aprotinin—and analyzed in two separate radioimmunoassay runs.

The effect of freezing and thawing on the C-peptide concentration was tested in the QC5078, QC5078.12, and QC5178 pools by collecting duplicate vials of each pool after freezing to −70 °C and thawing to room temperature for up to five times. After the required number of freeze–thaw cycles, the vials were again stored frozen, thawed simultaneously, and assayed in the same run by the modified method.
Modified C-Peptide Method

Pipette two 100-µL aliquots of the C-peptide calibrators (0 to 1000 pmol/L, prepared in a charcoal-stripped human serum base pool) into 12 × 75 mm glass tubes. Pipette four 100-µL aliquots of the zero calibrator for NSB correction to be applied to each calibrator. Pipette two 100-µL aliquots of each unknown and quality-control sample into glass tubes for unknown calculations. Pipette two 100-µL aliquots of each unknown and quality-control sample into glass tubes for NSB calculations. Pipette 100-µL aliquots of the M1230 antiserum (diluted 30 000-fold from stock with FAM) into all tubes except those for NSB calculations. Vortex-mix the contents of each tube, cover with paraffin film, and incubate all tubes for 24 h in a refrigerator at 4 °C. Pipette 100-µL aliquots of the 125I-labeled Tyr-C-peptide into all of the aforementioned tubes. Pipette six 100-µL aliquots of the 125I-labeled Tyr-C-peptide into empty glass tubes for measurement of total counts. Vortex-mix, cover, and incubate all tubes as above. Add 1.60 mL of 880 mL/L ethanol at room temperature to each tube, except those for total counts. Vortex-mix, then centrifuge all tubes together (2000 × g, 10 min). Carefully decant the supernatants into a waste beaker and blot the last drop from the lip of the tube with absorbent paper. Wipe excess liquid from the outer surface of the tubes with clean paper towels. Count the radioactivity of all tubes with a gamma counter. Subtract the mean NSB counts/min values from the mean counts/min values of their respective calibrator, unknown, or quality-control samples. Divide the NSB-corrected counts/min by the mean total counts/min and multiply by 100 to obtain the percent binding. Construct a calibration curve of percent binding vs C-peptide concentrations in the calibrators. Re-assay samples, diluted twofold with a C-peptide-free base pool, if any of the replicates are >800 pmol/L. If samples are paired—for example, fasting and stress—both pairs should be repeated at twofold dilution in the same run. If the interpolated concentration of any diluted replicate is still >800 pmol/L, re-assay the samples after fourfold dilution.

Results

Figure 1 illustrates the effect of different concentrations of ethanol on the percent binding of antibody-bound C-peptide and the necessity of correcting bound/total ratios for NSB. Bound/total ratios for pools QC5078.6 and QC5078.12 increased by ~8% to 10% as the ethanol concentration was changed from 800 to 960 mL/L. At the same time NSB/total ratios increased by ~30–35%, confirming the need to closely monitor NSB.

The NOVO and the modified protocols involving the separation of bound and free C-peptide and subsequent steps were compared by analysis of variance after 10 or 11 analytical runs (Table 1). The recommended NOVO protocol did not seem to result in better precision for B/T ratios of C-peptide calibrators. Precision was improved by using the modified method for among-run and total CV estimates at C-peptide concentrations <1000 pmol/L. The lower CVs for the modified method at the 100 pmol/L C-peptide concentration probably are statistical anomalies, because they are much lower than the other adjacent concentrations.

Calibration curves with C-peptide calibrators prepared in NaFAM or QC5078 (base pool) show a significant matrix effect at high C-peptide concentrations (Figure 2). NSB-corrected B/T values were higher when calibrators were prepared in NaFAM rather than in serum, thus causing an overestimation (positive bias) of interpolated C-peptide in serum samples when the NaFAM matrix was used.

Testing the thermal stability of C-peptide in pooled normal human sera showed that, even at −20 °C, some instability of the peptide was detectable without aprotinin (Figure 3). The decay curves for storage at 4, 23, or 37 °C were similar, with or without aprotinin. Significant losses of C-peptide were evident at room temperature (23 °C) and above. Thus samples had to be kept frozen at a temperature below −20 °C until just before analysis. We saw no significant changes in C-peptide concentrations after as many as five freeze–thaw cycles of pools QC5078, QC5078.12, and QC5178.

Table 2 gives a comparison of the analytical recovery of C-peptide in serum pools QC5078, QC5078.6, and QC5078.12 when estimated with calibrators prepared in NaFAM or serum. Confirmation of the C-peptide removal in the charcoal-stripped base pool (QC5078) was obtained, as the C-peptide concentration was found to be essentially zero when measured with calibrators prepared in either matrix. Estimated C-peptide in the analytical-recovery samples at concentrations of 120 pmol/L or lower were comparable when calibrators in either matrix were used. When C-peptide was added to QC5078 to give final concentrations of 200 or 300 pmol/L, however, the use of NaFAM-based calibrators resulted in a severe positive bias—that is, 182% and 204%.
recoveries, respectively. In contrast, the use of serum-based calibrators yielded more accurate recoveries, with a slight decrease in recovery at lower C-peptide concentrations. During the ETDRS, when a patient’s C-peptide was estimated to be >800 pmol/L, the sample was diluted with the QC5078 base pool and analyzed in another run. This was necessary for 27% of all samples in the ETDRS.

Figure 4 shows the long-term performance and ruggedness of the modified C-peptide method for all three quality-control pools plotted as monthly means over a four-year period. The total CVs for the high-concentration pool (QC5178), the middle-concentration pool (QC5078.12), and the low-concentration pool (QC5078.6) for this period (89 runs) were 9.4%, 5.7%, and 7.4%, respectively.

In the ETDRS the distribution of C-peptide concentrations measured in “stress” samples was strikingly bimodal, suggesting a division of study patients into two groups, those with stress C-peptide ≤80 pmol/L and those with >80 pmol/L (8). Constellations of clinical characteristics were found that could serve as proxies for the stress C-peptide concentration. The mean (and SD and n) fasting and stress C-peptide concentrations were 18.7 (61.3, 308) and 13.5 (12.6, 309) pmol/L, respectively, for samples from patients whose stress C-peptide concentration was ≤80 pmol/L. The mean fasting and stress C-peptide values were 524 (417, 235) and 1072 (705, 236) pmol/L, respectively, for patients whose stress C-peptide concentration was >80 pmol/L.

Of the ETDRS patients for whom data were available, half had C-peptide concentrations of ≤25 pmol/L during fasting or stress and stress/fasting differences of ≤13.5 pmol/L.

Discussion

Given the slight plateau observed in the bound/total ratios at 880 mL/L ethanol concentration (but still giving >81% of the maximum binding), and NSB values of ≤10% (Figure 1), we chose to use 880 mL of ethanol per liter for the most efficient separation of bound from free tracer for use in
the modified method. The 950 mL/L ethanol concentration
recommended in the commercial method was unacceptable,
yielding NSB counts that exceeded the antibody-bound
counts. The modified protocol—that is, separation with 880
mL/L ethanol, decanting the supernate and blotting the
tubes after centrifugation, and no addition of NaOH to the
pellet—was judged to be equal to or better than the NOVO
protocol, as based on the within-run, among-run, and total
CV estimates shown in Table 1. One reported comparison of
commercial methods for C-peptide (15) found that the
NOVO kit produced overall CVs of 11.6% and 12.2% for
sample means of 60 and 513 pmol/L, respectively. At the
same approximate concentrations—50 and 500 pmol/L—the
modified method yielded CVs of 6.2% and 10.7%, respectively.
In addition, the use of fewer steps in the modified method
required less technician time and decreased the potential for
error or breakage. Because of the evidence for some instabili-
ity of serum samples at −20 °C in the present study (Figure
3) and in another study (16), the patients’ samples were
shipped from the clinical centers on solid CO2. After receipt,
the patients’ samples and the C-peptide quality-control
pools were stored at −70 °C until analysis.

The improvements to the commercial C-peptide method
were of considerable value when applied to the errors
samples. A previous evaluation study (15) of commercially
available C-peptide kits indicated the narrow analytical
range of the NOVO kit as a primary difference among the
evaluated kits. The excellent sensitivity of the modified
method was invaluable in the present study population of
patients with long-term diabetes, some of whom have very
little beta-cell function. The improved detection limit result-
ed from the use of serum-based calibrators to decrease the
well-known matrix influence that is observed in many
immunoassays, the correction for NSB in the computation of
the calibration curve and of the unknown samples, and the
more efficient differential precipitation step with 880 mL/L
ethanol. These corrections for NSB required more tubes per
sample in the protocol, thus decreasing the method’s through-
put, but the increase in accuracy and sensitivity
more than compensated for the extra time and tubes that
were required. Any sample with a C-peptide value >800
pmol/L was diluted with QC5078 and re-assayed, along with
its paired fasting or stress sample.

Perhaps the most dramatic improvement to the NOVO
protocol by the modified method was in the analytical
recovery data. The positive bias seen when calibrators were
prepared in defined matrix (NaFAM) was extremely high
(>180%) at C-peptide concentrations >200 pmol/L. The
effect of matrix bias was not examined in a previous
evaluation of C-peptide kits (15). Such a bias would tend to
overestimate not only the absolute C-peptide concentration
but, more importantly, the C-peptide increase after a carbo-
hydrate challenge. Also, because the bias was concentration
dependent, the greater the beta-cell secretory activity, the
greater the error would be at the higher C-peptide concen-
trations. Such magnitudes of variable error would make multiyear study data, such as in ETDRS, very difficult, if not
impossible, to interpret accurately. The modified method,
however, obtained improved analytical recoveries at all C-
peptide concentrations up to 300 pmol/L, the highest concen-
tration tested (Table 2). Having this degree of control over
the accuracy allows for comparing data early and late in the
study, especially when computing stress/fasting differences
in C-peptide concentrations.

While developing and implementing the C-peptide
method for the ETDRS, we recognized that it would be important
to obtain a C-peptide antiserum with minimal cross-reactivity
to proinsulin. This was the reason for obtaining antiserum
M1230, which is claimed to have ~10% cross-reactivity with
proinsulin (9). One C-peptide method comparison study (17)
showed that a method based on use of M1230 without prior
removal of proinsulin from the sample yielded lower values
for C-peptide concentrations in proinsulin-rich plasma ob-
tained from patients with insulinoma than did two other
methods where insulin antibodies were used for proinsulin
removal. Another study (18) measured C-peptide in patients
with Type I diabetes by two methods: one by the method of
Heding (10), after removing proinsulin by precipitating
anti-insulin antibody–proinsulin complexes with polyelec-
eny glycol before the C-peptide measurement, and the other
by the NOVO kit. The results were almost identical for the
two methods, confirming the low cross-reactivity of antise-
rum M1230 with proinsulin. Bulk quantities of M1230 were
purchased and stored to ensure the availability of this
antisera for the duration of the errors.

Table 2. Effect of Calibrator Matrix on the Analytical Recovery of C-Peptide

<table>
<thead>
<tr>
<th>Quality-control pool</th>
<th>Target value, pmol/L</th>
<th>Measured value, pmol/L</th>
<th>Recovery, %</th>
<th>Calibration matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td>QC5078</td>
<td>0</td>
<td>5.1 (4.6)</td>
<td>80</td>
<td>Serum-based</td>
</tr>
<tr>
<td>QC5078.6</td>
<td>60</td>
<td>57.3 (10.9)</td>
<td>74</td>
<td>Serum-based</td>
</tr>
<tr>
<td>QC5078.12</td>
<td>120</td>
<td>134 (22.2)</td>
<td>74</td>
<td>Serum-based</td>
</tr>
<tr>
<td>QC5078 + 20 d</td>
<td>200</td>
<td>369 (8.3)</td>
<td>32</td>
<td>Serum-based</td>
</tr>
<tr>
<td>QC5078 + 30 d</td>
<td>300</td>
<td>618 (159)</td>
<td>20</td>
<td>Serum-based</td>
</tr>
</tbody>
</table>

*a Number of determinations. b (mean – QC5078 mean)/target value] × 100. c Mean (and SD). d Microliters of 1000 pmol/L C-peptide calibrator added to the first

Fig. 4. Quality control during four years of method performance: monthly mean C-peptide concentrations for pools QC5178, QC5078.12, and QC5078.6 plotted vs the month of analysis.

Total coefficients of variation (CV) for QC5178, QC5078.12, and QC5078.6 were 9.4%, 5.7%, and 7.4%, respectively.
The importance of the ruggedness and reliability of an immunoassay such as C-peptide to its application in a multiyear clinical trial designed for evaluating selected populations cannot be overemphasized. The reliability of the data produced by the analytical method is critical for resolving the study questions. The analytical confidence for the first study sample must be equivalent to that for the last sample, and any analytical drifts in performance cannot be tolerated. Our studies with C-peptide methods resulted in the development of appropriate sample handling and storage conditions and of a modified C-peptide method demonstrating consistent performance for four years in a clinical trial study.

We thank Melanie Wilson (now deceased) for her excellent technical assistance during this project. We also thank the staff members at the three centers, mentioned above, that participated in this study. Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Dept. of Health and Human Services.

References