I evaluated the between-method variation of C-peptide determined with C-peptide radioimmunoassay kit reagents from Mallinckrodt, Behring, Diagnostic Products Corp., and Immuno Nuclear Corp., and antisera from Novo (M1230, M1221, and K6) and Cambridge Medical Diagnostics (299-029P). C-peptide concentrations corresponding to 0.6 nmol/L (as determined with M1230) ranged from 0.54 (by Immuno Nuclear Corp.) to 1.06 nmol/L (by M1221). Furthermore, pretreating plasma specimens with Polyethylene Glycol 6000 variably decreased the results. Several factors may contribute to the between-method variation of C-peptide analyses: differences in the specificity of antisera, the type of C-peptide preparation used as standard and tracer, liability of the immunoassay to nonspecific interference, and possibly the heterogeneity of C-peptide immunoreactivity in the specimens.

Additional Keyphrases: diabetes • proinsulin • diagnosis of insulinoma • cutoff values

The C-peptide of proinsulin is extensively determined, to measure endogenous insulin secretion in diabetes mellitus (1–4) and, with determinations of proinsulin, to aid in the diagnosis of insulinoma (5). Insulin and C-peptide are secreted simultaneously and in equimolar quantities into the portal blood. During the first-pass circulation, a considerable (and variable) proportion of the insulin is extracted by the liver. In contrast, practically all of the C-peptide secreted enters the systemic circulation intact. Most C-peptide is degraded in the kidney, a small proportion of the intact peptide being excreted in the urine. Determination of C-peptide has several advantages over insulin immunoassays in assessment of endogenous insulin secretion. Because it avoids the first-pass hepatic metabolism, the concentration of C-peptide in peripheral blood is a more reliable measure of insulin secretion than is the measured concentration of insulin. Moreover, C-peptide immunoassays do not measure exogenous insulin and are not subject to severe interference from insulin antibodies induced by therapy with insulin (4).

Despite the advantages of C-peptide determination, its clinical application requires the resolution of several problems. The use of discriminating limits, in diagnosis or therapy, assumes adequate standardization. However, the various antisera are known to differ in their antigenic domains (6), and different materials have been used as standards, intact human C-peptide as well as various C-peptide derivatives. Because circulating C-peptide immunoreactivity has several sources (7), it cannot necessarily be quantitatively equated with human C-peptide (i.e., human proinsulin fragment 33–63). Taken together, the differences contribute to the variation of C-peptide concentrations as measured with different immunoassays. Even minor cross-reactivity of C-peptide antisera with human proinsulin may significantly interfere in C-peptide immunoassays if residual proinsulin secretion persists in a diabetic patient who has circulating insulin antibodies induced by insulin therapy. In such a case proinsulin may be retained in blood as immunocomplexes consisting of proinsulin and insulin antibodies. If the concentration of such immunocomplexes is high enough, they may bring about spuriously increased concentrations of immunoreactive C-peptide (8, 9).

Among the diversity of immunoassays for C-peptide measurements, one extensively used method was based on C-peptide antisera (M1230) from Novo. The validity of this immunoassay for clinical applications has been verified in several clinical studies (10–15). This antisera is, however, no longer commercially available. The aim of the present study was to investigate the possibility of replacing this antisera with some of the presently available antisera with no substantial worsening of the performance of the C-peptide radioimmunoassay. Furthermore, I examined several presently available C-peptide radioimmunoassay kits to see whether results would be so immunoassay-specific as to affect discriminating limits (cutoff values) used in making therapeutic decisions.

Materials and Methods

Materials. C-peptide antisera M1230, M1221, and K6 (Novo, Bagsvaerd, Denmark) and no. 299-029P (Cambridge Medical Diagnostics, Billerica, MA) were used in C-peptide immunoassays as described earlier (9), with some modifications. Before assay, plasma samples were diluted 10-fold in 40 mmol/L phosphate buffer (pH 7.4) containing 6 g of NaCl and 60 g of human albumin (Behring, Marburg, F.R.G.) per liter. I diluted the antisera in 40 mmol/L phosphate buffer containing 1 g of human albumin per liter to give working dilutions of 1:30 000 (M1230), 1:18 000 (M1221), 1:9000 (K6), and 1:40 000 (299-029P). The tracer, human Tyr-C-peptide labeled with 125I (Novo, cat. no. 111), was diluted in the same buffer as the antisera.

Procedures

Sequential assay. Mix, and incubate overnight at 4 °C, 100 μL of the diluted plasma specimens or human C-peptide standard (Novo, cat. no. 820) in original concentrations ranging from 0.01 to 0.5 nmol/L and 100 μL of the diluted antisera. On the following day, add 100 μL of the tracer reagent and incubate the reaction mixture overnight again at 4 °C. The next day, add 100 μL of 10 g/L human gammaglobulin solution (Finnish Red Cross, Helsinki, Finland) and 400 μL of 250 g/L polyethylene glycol solution (PEG-6000; Fluka, Buchs, Switzerland) mix thoroughly, and centrifuge. Aspirate and discard the supernate, then count the radioactivity.

Equilibrium assay. I also used the antiserum 299-029P in an equilibrium assay, mixing the specimen or the standard,
antiserum, and radiolabeled C-peptide simultaneously and then separating the antibody-bound and free radiolabeled C-peptide after a single overnight incubation.

**Diluents.** I used three types of diluents for the standards, to determine whether any kind of matrix effect would affect the reaction kinetics between the standard, tracer, and antiserum: I investigated the phosphate buffer described above containing human albumin 60 g/L; phosphate buffer containing human plasma (100 mL/L) from diabetic patients devoid of endogenous C-peptide; and phosphate buffer supplemented with human plasma (100 mL/L) that had been treated with charcoal suspension to remove endogenous C-peptide and other potentially interfering substances of low molecular mass.

**Kits.** I used C-peptide radioimmunoassay kits from the following manufacturers: Immuno Nuclear Corp., Stillwater, MN (cat. no. 4500); Diagnostic Products Corp., Los Angeles, CA (cat. no. KPEDI); Behringwerke, Frankfurt am Main, F.R.G. (cat. no. OCSK 03); and Mallinckrodt Diagnostica, Dietzenbach, F.R.G. (cat. no. 3791). In all cases I followed the detailed instructions provided by the manufacturers for the immunoassay procedures.

**Specimens**

Plasma specimens were obtained from type I and II diabetic subjects (28% and 72% of whom were receiving glucagon and a breakfast meal, respectively, to stimulate insulin secretion). Samples were drawn in "Vacutainer" evacuated blood-collection tubes containing lithium heparin as anticoagulant (Becton Dickinson, Meylan Cedex, France). Tubes were placed in crushed ice immediately after the blood specimens were drawn. The plasma was separated without delay and stored at -20 °C until analyses. In several methods the plasma specimens were pretreated with polyethylene glycol solution to precipitate insulin antibodies before C-peptide assays. This was done by thoroughly mixing equal amounts of plasma specimen and 250 g/L polyethylene glycol solution (Fluka) at room temperature. The PEG-treated samples were centrifuged immediately after the addition of PEG (1900 × g, 20 min). The clear supernates were used for C-peptide determination.

**Statistical methods.** Linear regression and correlation analyses were used for the between-method comparisons. Student's paired t-test was used to assess the significance of the mean of the differences between paired measurements. Within-assay reproducibility was evaluated by determining the standard deviation of the duplicate measurements according to the formula SD = √(Σd²)/2N, in which d is the absolute difference between paired measurements and N is the number of pairs of measurements.

**Results**

C-peptide radioimmunoassay based on the use of antiserum M1230 was chosen for the reference method in the between-method comparisons (Figure 1, Table 1). The intraassay standard deviation of paired measurements was 0.04 nmol/L. The between-assay coefficient of variation was 7.3% at a C-peptide concentration of 0.76 nmol/L (n = 43).

C-peptide concentrations measured with the assay based on the use of antiserum 289-029P from Cambridge Medical Diagnostics agreed best with those measured with the assay in which antiserum M1230 was used (Figure 1, Table 1). C-peptide concentrations were somewhat lower in PEG-pre-treated plasma samples than in untreated samples. However, the difference was not statistically significant.

The performance of this method was further evaluated. Plasma samples devoid of endogenous C-peptide were supplemented with the C-peptide standard used in the immunoassay. Analytical recovery of C-peptide ranged between 94% and 117%. The method was found to be linear for plasma dilutions ranging from five- to 200-fold. Use of the equilibrium assay resulted in a loss of sensitivity as compared with the sequential assay (Figure 2). In a batch of plasma samples with C-peptide concentrations ranging from 0.21 to

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Fig. 1. Results of the interassay comparisons of C-peptide immunoassay reagents: C-peptide concentrations obtained with antiserum M1230 vs the other methods used

*INC, Immuno Nuclear Corp.; DPC, Diagnostic Products Corp.; CMD, Cambridge Medical Diagnostics. Lines are the lines of equality, for comparison*

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Table 1. Results of the Inter-Method Comparison of the C-Peptide Radioimmunoassays

<table>
<thead>
<tr>
<th>Comparison method*</th>
<th>Slope (coeff.)</th>
<th>y-intercept, nmol/L</th>
<th>r</th>
<th>n</th>
<th>C-peptide measurement corresponding to 0.6 nmol/L (M1230, Novo): mean and 95% confidence interval, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>K6 (Novo)</td>
<td>0.98</td>
<td>0.19</td>
<td>0.99</td>
<td>13</td>
<td>0.78 (0.74–0.82)</td>
</tr>
<tr>
<td>K6 (Novo)+ PEGb</td>
<td>1.12</td>
<td>0.10</td>
<td>0.98</td>
<td>13</td>
<td>0.78 (0.72–0.84)</td>
</tr>
<tr>
<td>M1221 (Novo)</td>
<td>0.77</td>
<td>0.59</td>
<td>0.89</td>
<td>12</td>
<td>1.06 (0.99–1.13)</td>
</tr>
<tr>
<td>M1221 (Novo) + PEG</td>
<td>1.05</td>
<td>0.07</td>
<td>0.97</td>
<td>13</td>
<td>0.70 (0.63–0.77)</td>
</tr>
<tr>
<td>Mallinkrodt</td>
<td>1.29</td>
<td>-0.07</td>
<td>0.97</td>
<td>28</td>
<td>0.70 (0.66–0.74)</td>
</tr>
<tr>
<td>Mallinkrodt + PEG</td>
<td>1.27</td>
<td>-0.09</td>
<td>0.94</td>
<td>33</td>
<td>0.68 (0.63–0.73)</td>
</tr>
<tr>
<td>DPC + PEG</td>
<td>1.19</td>
<td>0.08</td>
<td>0.94</td>
<td>33</td>
<td>0.80 (0.72–0.88)</td>
</tr>
<tr>
<td>Behring</td>
<td>1.60</td>
<td>0.09</td>
<td>0.85</td>
<td>29</td>
<td>1.04 (0.91–1.17)</td>
</tr>
<tr>
<td>INC</td>
<td>0.99</td>
<td>-0.06</td>
<td>0.99</td>
<td>31</td>
<td>0.54 (0.49–0.59)</td>
</tr>
<tr>
<td>INC + PEG</td>
<td>0.94</td>
<td>-0.02</td>
<td>0.97</td>
<td>31</td>
<td>0.54 (0.50–0.58)</td>
</tr>
<tr>
<td>CMD</td>
<td>1.02</td>
<td>0.01</td>
<td>0.98</td>
<td>25</td>
<td>0.63 (0.58–0.68)</td>
</tr>
<tr>
<td>CMD + PEG</td>
<td>1.29</td>
<td>-0.05</td>
<td>0.98</td>
<td>23</td>
<td>0.71 (0.67–0.75)</td>
</tr>
</tbody>
</table>

*The comparison method is that involving antisera M1230, C-peptide standard, and radiolabeled C-peptide from Novo.

b + PEG plasma samples pretreated with Polyethylene Glycol 6000 before analysis.

DPC, Diagnostic Products Corporation C-peptide kit.

INC, Immuno Nuclear Corporation C-peptide kit.

CMD, Cambridge Medical Diagnostics, antisera 295-023P.

2.4 nmol/L (M1230), mean C-peptide concentrations as measured with the equilibrium assay were significantly higher than with the sequential assay: 0.92 (SD 0.82) vs 0.75 (SD 0.76) nmol/L (P <0.001, n = 11). This difference could not be explained by any differences of the reaction matrices between the standard and the specimens, because the standard curves were unaffected by the addition of either type of human plasma tested.

The within-assay standard deviation of the paired measurements was 0.05 nmol/L. The between-assay CV was 5.7% at a C-peptide concentration of 0.91 nmol/L (n = 43).

The use of antisera M1221 or K6 (Novo) yielded significantly (P <0.001) higher values for immunoreactive C-peptide, especially when antisera M1221 was used without PEG-pretreatment of plasma specimens (Figure 1, Table 1). PEG-pretreatment of plasma specimens had no significant effect on C-peptide concentrations as measured with use of antisera K6. Significantly (P <0.001) lower C-peptide concentrations were found after PEG-pretreatment when antisera M1221 was used.

Figure 1 and Table 1 summarize the results of the intercomparison of the commercial C-peptide radio immunoassay kits. The value 0.6 nmol/L measured with M1230, the limit used for the classification of diabetic patients (13, 15), corresponded to 1.04 (SD 0.13) nmol/L with the method of Behring. At high C-peptide concentrations the method of Diagnostic Products Corporation also yielded higher values for C-peptide, whereas at lower C-peptide concentrations the difference was minor, or even reversed. C-peptide concentrations measured with the Mallinkrodt method were also higher than with antisera M1230 (P <0.05) but lower with the Immuno Nuclear Corp. kit (P <0.01). (Later, the composition of the Immuno Nuclear Corp. kit was modified by the manufacturer; C-peptide concentrations measured with the new version of the kit were found to be about 20% higher. Comparison could not be made, however, against antisera M1230 because of exhaustion of our supplies.) C-peptide concentrations measured with the method of Mallinkrodt were slightly lower after PEG-pretreatment of plasma specimens (P <0.05). No such difference was found with the Immuno Nuclear Corporation kit.

The within-assay standard deviation of paired measurements ranged between 40 pmol/L (the Mallinkrodt kit) and 140 pmol/L (radioimmunoassay based on the antisera K6, Novo) in the various methods I examined.

Discussion

The variation in C-peptide concentrations as measured with the various radioimmunological methods clearly demonstrates the possibility of confusion in interpretation of results of C-peptide analyses. Similar findings are presented in two previous studies on the subject (16, 17). One of the most extensively used clinical applications of C-peptide analyses is the estimation of endogenous insulin secretory reserves in the evaluation of the need for exogenous insulin in diabetes mellitus. For C-peptide measurements with antisera M1230 in a glucagon test (10), a discriminating limit of 0.6 nmol/L has been proposed as a criterion of insulin deficiency necessitating insulin therapy (13, 15).

![Fig. 2. Standard curves obtained with the antisera of Cambridge Medical Diagnostics (295-023P) and radiolabeled C-peptide and C-peptide standard from Novo.](image-url)
Table 2. Characterization of C-Peptide Radioimmunoassay Reagents

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Standard</th>
<th>Antiserum, species, and % cross-reactivity*</th>
<th>Tracer</th>
<th>Sepn. of free and antibody-bound tracer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novo</td>
<td>Human C-peptide</td>
<td>M1230 Guinea pig (12%) M1221 Guinea pig (80%) K6 Rabbit (75%)</td>
<td>Human Tyr-C-peptide</td>
<td>PEG 6000</td>
</tr>
<tr>
<td>Mallinckrodt</td>
<td>Human C-peptide</td>
<td>Rabbit (36%)</td>
<td>Tyr-C-segment</td>
<td>Double-antibody pptn.</td>
</tr>
<tr>
<td>DPC</td>
<td>Human C-peptide</td>
<td>Goat (15%)</td>
<td>C-segment</td>
<td>Double-antibody pptn.</td>
</tr>
<tr>
<td>Behring</td>
<td>Human C-peptide</td>
<td>Rabbit (12%)</td>
<td>p-Hydroxyphenylpropionate–C-peptide</td>
<td>PEG 6000</td>
</tr>
<tr>
<td>INC</td>
<td>Human Tyr-C-peptide</td>
<td></td>
<td>Human Tyr-C-peptide</td>
<td>Double-antibody pptn.</td>
</tr>
</tbody>
</table>

*Cross reactivity with human proinsulin (molar basis). The Cambridge Medical Diagnostics guinea pig C-peptide antiserum 299-029P cross-reacts with human proinsulin by 2.6%.

Abbreviations as in Table 1.

Such limits cannot be applied without a reference to the method used for measuring the C-peptide concentrations. Even the use of the same reagents does not necessarily ensure equality of results, because even minor variations in the assay procedure may alter the results, as demonstrated above in the difference between the sequential and equilibrium assays. In the study of Caygill et al. (18) the use of identical standard in various C-peptide radioimmunoassays in different laboratories was shown not to ensure the transferability of the results of C-peptide measurements. Occasionally, commercial C-peptide radioimmunoassay kit reagents may be altered by the manufacturers, and this should be taken into account when comparing the results obtained with two versions of a commercial radioimmunoassay kit, even though the two may have identical trade names.

Currently used methods differ in several respects. Human C-peptide (human proinsulin fragment 33-63) and C-peptide derivatives have been used as standards (Table 2). The reported molar cross-reactivity with proinsulin of the antisera used in this study varies between 2.6% (299-029P, Cambridge Medical Diagnostics) and 80% (M1221, Novo). In diabetic patients as well as in normal subjects the insulin secretion has been shown to consist partially of proinsulin (6, 12, 19). The presence of insulin antibodies induced by insulin therapy may lead to retention of proinsulin, resulting in concentrations high enough to interfere significantly in a C-peptide radioimmunoassay (6). The prevalence of such a situation is, however, probably low if an antiserum with high specificity is used. Insulin antibodies can be extracted with polyethylene glycol, or proinsulin can be extracted with solid-phase-coupled insulin antibodies (9), but either technique makes the radioimmunoassay more cumbersome and so less suited for routine analyses, and may hamper analytical precision.

In conclusion, the present data demonstrate the between-method variation of different C-peptide radioimmunoassays. Of the immunoassays I tested, the most feasible choice for replacing the currently unavailable C-peptide antiserum M1230 appears to be antiserum 299-029P (Cambridge Medical Diagnostics). The findings emphasize the necessity to define the C-peptide concentration limits applied for therapeutic decisions specifically for the method used to measure C-peptide.

References