Feasibility of Standardization of Serum C-Peptide Immunoassays with Isotope-Dilution Liquid Chromatography–Tandem Mass Spectrometry, Diego Rodríguez-Cabaleiro, Dietmar Stöckl, Jean M. Kaufman, Tom Fiers, and Linda M. Thienpont

Background: Serum C-peptide concentrations reflect pancreatic function in different clinical and diagnostic settings; however, the utility of C-peptide testing is limited by the lack of standardized commercial immunoassays. Standardization can best be done by split-sample comparison with a hierarchically higher reference measurement procedure used with a set of native sera. For serum peptides, isotope-dilution liquid chromatography–mass spectrometry (ID-LC/MS) is recommended as a reference measurement procedure.

Methods: We evaluated the analytical performance characteristics of an ID-LC/tandem MS procedure for measurement of serum C-peptide after a 2-step solid-phase extraction. To investigate the feasibility of this procedure for use in standardization, we also performed a method comparison with 3 representative commercial assays.

Results: The ID-LC/tandem MS procedure showed maximum within-run, between-run, and total CVs on dedicated sera (C-peptide concentrations, 1.6 and 4.0 μg/L) of 2.1%, 2.5%, and 2.9%, respectively; an accuracy of 94.6%–104.1%; a minimum trueness of 98.1% (95% confidence interval, 96.2%–100.0%), and limits of quantification and detection of 0.15 and 0.03 μg/L, respectively. Deming linear regression analysis of the method-comparison data showed that the immunoassays correlated well with ID-MS and were specific, but lacked intercomparability and trueness. We propose that the deficiencies can be resolved by recalibration on the basis of the method comparison.

Conclusions: The ID-LC/tandem MS procedure is suitable for specific and accurate measurement of basal and stimulated serum concentrations of proinsulin C-peptide fragment 33–63 and is suitable for use in standardization of C-peptide immunoassays.

Measurement of C-peptide testing under controlled analytical conditions or interpretation of results against assay-specific reference intervals [see, for example, Refs. (2, 9, 11–13)]. To overcome these limitations, worldwide standardization of C-peptide immunoassays is needed (1, 14). This standardization can best be achieved by split-sample comparison with a hierarchically higher reference measurement procedure used with a set of native sera (15, 16). Such an assay method comparison documents both calibration status and specificity. In the case of C-peptide assays, standardization with assessment of specificity is particularly important because of the risk for cross-reactivity with proinsulin and the heterogeneity in circulating forms that react with antisera to a different extent. Isotope-dilution liquid chromatography–mass spectrometry (ID-LC/MS) is the recommended reference measurement procedure for serum peptides because it allows direct calibration with a primary calibrator and guarantees specific and accurate measurement of the analyte (15–17). The latter is defined as proinsulin C-peptide fragment 33–63 with the amino acid sequence EAEDLQVGQVELGGGPGAGSLQ with proinsulin and the heterogeneity in circulating mass (M₁) 3020.3. All diagnostic companies use this definition [see, for example, Refs. (18–20)].

Here we report on the validation of an ID-LC/tandem MS procedure suitable for measurement of basal and stimulated serum C-peptide concentrations. To investigate the feasibility of its use for the purpose of standardization, we performed a method-comparison study with 3 representative commercial assays.

The development of the basic ID-LC/tandem MS procedure (calibration, sample purification, chromatography, MS conditions) has been described elsewhere (21–23). For a detailed presentation of the experimental conditions and a representative chromatogram, see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue6. Briefly, we used C-peptide and its 4-Val analog (Bachem) as calibration material and internal standard, respectively. Calibration solutions contained 0.03 g/L bovine serum albumin and had a C-peptide concentration of 0.03 mg/L. We controlled all volumetric steps gravimetrically, so that the concentrations were accurately known to 4 significant figures. We processed between 0.3 (minimum) and 2.5 mL (maximum) of serum, depending on the sample size available. We purified the samples by a 2-step solid-phase extraction procedure (Sep Pak C₁₈ followed by Oasis® MCX; both from Waters). Chromatography was done with a Model 325 HPLC system (Kontron Instruments) equipped with a Hamilton PRP-3 column (50 x 2.1 mm (i.d.)). The HPLC column was connected to a VG Quattro II mass spectrometer (Micromass) operated in the negative electrospray ionization mode. Different from our previous reports (21–23), we used an energy of 80 eV for collision-induced dissociation and monitored the transitions of the doubly charged deprotonated molecular ions [M – 2H]²⁻ from m/z 1509.2 to 183.8 (C-peptide) and from 1517.1 to 183.8 (4-Val C-peptide). The experiments done for the investigation of...
the fragment ion at m/z 183.8 are discussed in the online Data Supplement.

The method comparison was done with the electrochemiluminescence immunoassay with the Modular Analytics E170 (Roche Diagnostics GmbH), the Immulite® 2000 C-peptide chemiluminescent enzyme immunoassay from Diagnostic Products Corporation (DPC), and the \(^{125}\)I-based IRMA-C-PEP from Cis bio international. All assays are calibrated against the WHO First International Reference Preparation (IRP) 84/510 (24, 25).

We used the following materials for evaluation of the performance characteristics of the ID-LC/tandem MS method: (a) 2 sera from a voluntary blood donation after overnight fasting and 60 min after glucose stimulation (concentrations, 1.6 and 4.0 \(\mu g/L\)) for imprecision; (b) 2 supplemented serum pools used in the method comparison as internal quality-control materials (concentrations, 4.1 and 14.8 \(\mu g/L\)), for imprecision; (c) a protease-free bovine serum albumin solution (70 \(g/L\) in a saline solution (9.0 \(g/L\) NaCl)) and a serum pool (baseline concentration, 1.16 \(\mu g/L\)), for imprecision; (d) 6 calibration mixtures, prepared from solutions containing 0.03 \(g/L\) bovine serum albumin and measured directly, and another 6 prepared from solutions containing 10 \(g/L\) albumin and measured after processing, to assess the stability of the calibrators; and (e) samples selected from the method-comparison study to evaluate interference, ion suppression, and the limits of detection (LOD) and quantification (LOQ).

For the method comparison, we used serum samples from 15 ambulatory patients (2 men and 13 women; age range, 18–64 years) from Ghent University Hospital. They had been subjected to an oral glucose (75 g) tolerance test after overnight fasting. The C-peptide tests were ordered by the endocrinologist for assessment of glucose tolerance, glucose handling, and \(\beta\)-cell function in persons with morbid obesity and/or other risk factors for glucose resistance and intolerance. Patient samples were handled according to the local Ethical Committee guidelines. Blood was drawn into Venosafe VF-106SAS tubes (Terumo) before the glucose load and 30, 60, 120, and 180 min after glucose loading. The collected blood was allowed to clot for at least 30 min and was centrifuged at 1500\(g\) for 10 min. The fasting blood samples were transported to the laboratory within 30 min after withdrawal, whereas the remaining samples were received within 30 min after the last collection. Analysis with the 3 immunoassays was started immediately after receipt of the last sample. The remaining serum was transported ice-cooled to the MS laboratory. There, the samples were immediately processed by solid-phase extraction. The evaporated extracts were stored between 2 and 8 °C until LC/tandem MS analysis on the next day.

The design of the experiments for the evaluation of the ID-MS performance characteristics and method comparison (number of experiments, measurement protocol, number of runs, and quality control) is described in detail in the online Data Supplement.

Statistical data analysis was done with CBstat. Model II ANOVA was used to calculate the within-run, between-run, and total CVs (%). Two-sided \(F\)- and \(t\)-tests (\(\alpha = 0.05\)) were used for analysis of the stability of the calibration solutions. Confidence intervals (95%, two-sided) were calculated for the trueness data. Deming regression was used for analysis of the method-comparison data.

The performance characteristics of the ID-LC/tandem MS procedure are summarized in Table 1. The maximum within-run, between-run, and total imprecision (expressed as CV) was 3.5%, 2.5%, and 3.8%, respectively. The accuracy ranged between 94.6% and 104.1%, and the

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<th>Table 1. Precision, accuracy, and trueness of the ID-LC/tandem MS procedure.</th>
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<td>Dedicated sera(^a)</td>
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<tr>
<td>1.6 (\mu g/L) (n = 11)(^c)</td>
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<td>4.0 (\mu g/L) (n = 11)</td>
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<td>Internal quality-control materials(^d)</td>
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<td>14.8 (\mu g/L) (n = 12)</td>
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<td>Albumin solutions (n = 5)</td>
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<td>Supplemented serum(^e) (n = 5)</td>
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\(^a\) CI, confidence interval.
\(^b\) Mean endogenous serum C-peptide concentration.
\(^c\) Number of analytical runs in which a sample was assayed in duplicate.
\(^d\) Mean C-peptide concentration in a supplemented serum pool.
\(^e\) The basal C-peptide concentration was 1.16 (95% CI, 1.11–1.21) \(\mu g/L\) (n = 4).
minimum trueness was 98.1% (95% confidence interval, 96.2%–100.0%). The LOD and LOQ were estimated at 0.03 and 0.15 µg/L, respectively. There was no indication of ion suppression or interference by analytes other than C-peptide/d4-Val10-C-peptide during LC/tandem MS measurements. Statistical analysis of the data obtained in the experiment to evaluate the stability of the calibration solutions revealed no significant differences (P = 0.63 in the F-test and 0.92 in the F-test).

The results of the method comparison study are summarized in Fig. 1 (individual assays are shown in panels A, B, and C; regression summary is shown in panel D). With ID-LC/tandem MS, the basal C-peptide concentrations in the 15 patient samples ranged from 0.5 to 3.7 µg/L, and in the stimulated concentrations ranged from 1.2 to 9.5 µg/L. The Deming regression analysis indicated that the commercial assays considerably overestimate the C-peptide concentrations in a proportional way (slopes significantly >1) and to a different extent (slopes: Cis bio = 1.90; Roche = 1.74; DPC = 1.53). In addition, we documented a statistically significant negative intercept for the Cis bio assay. This interassay discrepancy documents the fact that calibration with a common calibrator (IRP 84/510) did not provide comparability of the measures. On the other hand, all immunoassays showed excellent correlation coefficients (Roche, r = 0.9914; Cis bio, r = 0.9889; DPC, r = 0.9815) and low standard errors of the estimates (SSE = 0.46–0.59 µg/L). The magnitude of SSE is nearly entirely the result of the combined imprecision of the immunoassay and the ID-LC/tandem MS measurement procedure. This is in contrast to the suggested differences in specificity of different antisera (10). The high correlation coefficients and low SSE values are a good basis for recalibration of the investigated immunoassays by use of the regression equations of the method comparison.

In conclusion, we developed an ID-LC/tandem MS procedure with sufficient LOD/LOQ and specificity for interference-free measurement of basal and stimulated serum concentrations of proinsulin C-peptide fragment 33–63. The internal validation data documented the compensating effects of ID for eventual losses, degradation, or incomplete recovery of C-peptide, which is necessary for a reference measurement procedure. The method comparison showed good specificity of the immunoassays tested, but a lack of intercomparability and trueness. The method comparison demonstrated, however, the feasibility of assay standardization by use of regression equations. For this study, we used sera from patients subjected to stimulation tests, but other applications might require different types of samples.

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References

15. Stöckl D, Franzini C, Kratochvil J, Middle J, Ricos C, Thiennpont LM. Current...
Thyroglobulin (Tg) Recovery Testing with Quantitative Tg Antibody Measurement for Determining Interference in Serum Tg Assays in Differentiated Thyroid Carcinoma, Adrienne C.M. Persoon,1 Thera P. Links,2 Jürgen Wilde,2† Wim J. Sluiter,1 Bruce H.R. Wolfenbuttel,1 and Johannes M.W. van den Ouweland2† (1 Department of Endocrinology, University Medical Centre Groningen, Groningen, The Netherlands; 2 Nichols Institute Diagnostics, Bad Vilbel, Germany; 3 Canisius-Wilhelmina Medical Centre, Department of Clinical Chemistry, Nijmegen, The Netherlands; † current affiliation: R-biopharm, Darmstadt, Germany; * address correspondence to this author at: Canisius-Wilhelmina Medical Centre, Department of Clinical Chemistry, Weg door Jonkerbos 100, 6500 GS Nijmegen, The Netherlands; fax 31-24-3658671, e-mail j.v.d. ouweland@cwz.nl)

Background: Thyroglobulin (Tg) measurements are complicated by interference from Tg autoantibodies (TgAbs) or heterophilic antibodies (HAMAs). We used a new automated immunochromiluminometric assay (ICMA) with Tg recovery (TgR) on the Nichols Advantage® platform to reassess the clinical utility of recovery testing in detecting interference in serum Tg measurement in patients with differentiated thyroid carcinoma.

Methods: We used 2 TgAb methods to detect Tg measurement interference with TgR and quantitative TgAb measurement in sera from 127 patients. In a limited number of samples, we used an RIA as comparison method because it appeared to be minimally affected by TgAb.

Results: Prevalence of TgAbs was 13% (17 of 127) in either 1 or both TgAb assays. A compromised TgR (<70%) corresponded with TgAb positivity in either TgAb assay for 10 of 11 samples (91%), whereas a normal TgR (≥70%) corresponded with TgAb negativity in both assays for 95 of 101 samples (94%). In 6 TgAb-positive sera with TgR within the reference interval, there were no discrepancies between RIA and ICMA results. We obtained discordant RIA and ICMA results for 6 of 9 TgAb-positive sera with decreased TgR. In 1 TgAb-negative sample, the Tg result was falsely increased because of interference by HAMAs, as shown by an overrecovery of 126%.

Conclusions: The Nichols Advantage TgR assay is a valuable complementary method to overcome the technical problem of interference by TgAbs or HAMAs in TgAb assays. Further studies are needed to confirm the potential added value of this TgR assay.

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Serum thyroglobulin (Tg) measurements play a key role in the postsurgical follow-up of patients with differentiated thyroid cancer (DTC) (1), but these measurements are severely hampered by the presence of Tg antibodies (TgAbs), which can cause under- or overestimation of Tg concentration depending on the Tg assay format (2,3). The National Academy of Clinical Biochemistry guideline (4) recommends the use of sensitive TgAb immunoassays to detect TgAbs in favor of Tg recovery (TgR) testing because TgR testing fails to differentiate TgAb-positive and -negative sera (5,6). The differences in immunoreactivity between endogenous Tg and Tg added to patient serum, as well as the amount of Tg added and the duration of incubation, all appear critical to the TgR result (5,7). However, limitations of TgAb testing are also recognized: TgAb concentrations do not correlate with the degree of interference (3,8); TgAb positivity does not indicate interference per se; substances other than TgAbs can interfere with Tg measurement (9); and TgAb detection is strongly method dependent (10).

A reliable hallmark of TgAb interference is the presence of RIA/immunometric assay discordance (4,10), but intermethod comparisons are impractical because few RIAs are available (10). Therefore, the technical problem of TgAb interference in Tg measurements has not been overcome. The release of a new TgR assay enabled us to reassess the clinical utility of recovery testing in detecting interference in serum Tg measurements by comparing the TgR assay with a quantitative TgAb test and the methodologic benchmark for TgAb interference, RIA/immunometric assay discordance (10), in relation to the clinical status of the patient.

We collected sera from 127 patients with DTC undergoing thyroid hormone suppression therapy who visited our outpatient clinic between May and September 2003. No evidence of disease was defined as absence of clinical, scinti-