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Measurement of Thyroglobulin by Liquid Chromatography–Tandem Mass Spectrometry in Serum and Plasma in the Presence of Antithyroglobulin Autoantibodies

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BACKGROUND: Measurement of serum thyroglobulin (Tg) is used to monitor patients after treatment for differentiated thyroid carcinoma (TC). Difficulty in using Tg as a biomarker of the recurrence of TC in many patients stems from the presence of endogenous anti-Tg autoantibodies (Tg-AAbs), which can interfere with immunoassays (IAs) and cause false-negative results.

METHODS: We enriched Tg from serum samples using rabbit polyclonal anti-Tg antiserum and protein precipitation. Unrelated proteins were partially depleted in the process. Enriched proteins were then denatured, reduced, and digested with trypsin after the addition of a winged internal standard peptide. A Tg-specific tryptic peptide was purified by immunoaffinity extraction and analyzed by 2-dimensional LC-MS/MS. Instrument cycle time was 6.5 min per sample.

RESULTS: The lower limit of quantification was 0.5 ng/mL (0.76 fmol/mL dimer). Total imprecision of triplicate measurements in serum samples over 5 days was <10%. Comparison with a commercial IA using serum samples free of Tg-AAb (n = 73) showed Deming regression, IA = 1.00 * LC-MS/MS − 2.35, r = 0.982, standard error of the estimate ($S_{y|x}$) = 9.52. In a set of Tg-AAb–positive samples that tested negative for Tg using IA (n = 71), concentrations determined by LC-MS/MS were ≥0.5 ng/mL in 23% of samples (median 1.2, range 0.7–11 ng/mL).

CONCLUSIONS: The introduced method has acceptable performance characteristics for use in clinical diagnostic applications. The most substantial disagreement between methods was observed in Tg-AAb–positive samples with concentrations <2 ng/mL (determined with LC-MS/MS). The affinity-assisted enrichment strategy used for Tg in this method should be applicable to other biomarkers that have endogenous autoantibodies.

Measurement of thyroglobulin (Tg)[5] is commonly used for the follow-up of patients treated for differentiated thyroid carcinoma (TC). Because thyroid tissue is the only source of Tg, after total thyroidectomy and radioactive ablation, serum concentrations of Tg should decrease to very low or undetectable. A rise in the serum concentration of Tg indicates cancer recurrence *(1, 2)*. In a retrospective assessment of the value of multiple potential markers of the recurrence of TC, posttreatment Tg concentration was found to be the strongest independent predictor of recurrence *(3)*. The presence of endogenous anti-Tg autoantibodies (Tg-AAbs) can mask the epitopes used by reagent antibodies in immunoassays for Tg, leading to falsely negative results *(4–7)*. Stokinger and Heidelberger *(4)* first described Tg autoantibodies. Active research of Tg-AAbs began >50 years ago *(8)*, but to date there are no commercial immunoassays available that can overcome the interference of Tg-AAbs in testing for Tg. To gauge the reliability of Tg measurements by immunoassay (IA), it is common practice to test every sample analyzed for Tg for the presence of Tg-AAbs and perform a Tg recovery test in samples testing positive for Tg-AAbs, although this approach can still miscategorize false negatives as true negatives *(9)*.

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[5] Nonstandard abbreviations: Tg, thyroglobulin; TC, differentiated thyroid carcinoma; AAb, autoantibody; IA, immunoassay; LC-MS/MS, liquid chromatography–tandem mass spectrometry; IAE, immunoaffinity enrichment; LOQ, limit of quantification; 2D, two-dimensional; IS, internal standard; FA, formic acid; DOC, sodium deoxycholate; LOD, limit of detection; AAE, affinity-assisted enrichment; $S_{y|x}$, standard error of the estimate.

It has been hypothesized that there might be a causal, pathophysiological link between thyroid autoimmunity and the development of TC *(9, 10)*. Importantly, approximately 25% of patients with TC and ≤10% of individuals without TC are positive for Tg-AAbs. Unfortunately, the presence of Tg-AAbs in these samples can interfere with quantification and lead to missed diagnoses of cancer recurrence. The only currently available methodology that could completely eliminate interference of Tg-AAbs with the measurement of Tg appears to be mass spectrometry *(11)*.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) has been increasingly used for quantifying peptides and proteins in biological samples *(11–14)*. Commonly used sample preparation techniques that do not include targeted enrichment of analyte often cannot provide the necessary selectivity for highly complex samples such as protein digests, whereas immunoaffinity enrichment (IAE) can greatly enhance the detection and quantification of proteins and peptides *(13)*.

LC-MS/MS has previously shown promise in the measurement of serum concentrations of Tg. A previously described method used proteolytic digestion, which cleaves serum proteins into peptides, followed by enrichment of Tg-specific peptides using antipeptide antibodies and quantitative analysis of the peptides with nano-LC-MS/MS *(11, 12)*. It has been suggested that the limit of quantification (LOQ) required for reliable detection of the recurrence of TC should be approximately 1 ng/mL *(3)*, whereas the LOQ of the previous method was 3 ng/mL *(12)*. In addition, the previously published method had limited throughput and involved nano-flow liquid chromatography.

Our goal was to develop an analytically sensitive, robust method for quantification of Tg in serum and plasma samples that would overcome interference of Tg-AAbs and evaluate its performance. The method we have developed uses Tg enrichment, proteolytic digestion, IA enrichment of the targeted peptide, 2-dimensional (2D) chromatographic separation, and MS/MS detection. The method was fully validated according to CLSI guidelines and applied to the analysis of clinical samples.

Materials and Methods

Detailed methods are provided in Supplemental Materials, which accompanies the online version of this article at http://www.clinchem.org/content/vol59/issue6.

PREPARATION OF REAGENTS, STANDARDS, AND QC SAMPLES

We purchased calibration standards containing Tg from Beckman Coulter and rabbit polyclonal anti-Tg antibody from Covance. Serum QC samples were pooled human serum samples. Internal standard (IS) winged peptides comprised the sequence PVPESK*VIFDANAPV*AVR*SKVPDS (V* [13C5,15N], mass shift 6 Da; RS Synthesis). We purchased trypsin (purity 99%, activity 15 000 BAEE units/mg protein), formic acid (FA), dithiothreitol, and sodium deoxycholate (DOC) from Sigma-Aldrich. All other reagents were of highest purity commercially available. Solvents were of HPLC grade, purchased from JT Baker.

CONJUGATION OF ANTIBODY TO MAGNETIC BEADS

Custom polyclonal rabbit antipeptide antibody (Covance) was conjugated to Tosyl activated magnetic beads (DynaBeads M280, Life Technologies) according to the manufacturer's recommendations.

SAMPLE PREPARATION

We prepared samples on a liquid handler (epMotion, Eppendorf). Rabbit anti-Tg antibody (300 ng) was added to serum or plasma (500 μL); samples were incubated; and then proteins were precipitated with saturated ammonium sulfate solution. After centrifugation (14 000*g* for 5 min), we reconstituted the precipitates with water (300 μL), added internal standard (10 μL, concentration 10 pg/μL), and reduced the samples at 60 °C. Samples were diluted in NH_4HCO_3 and digested with trypsin (40 μg) for 4 h at 37 °C. Preliminary experiments showed that DOC was critical for recovery of the targeted peptide. We optimized the denaturing, reduction, and digestion conditions to ensure rapid, complete, and reproducible digestion of Tg. Preliminary experiments showed no effect of cysteine alkylation on the recovery of the targeted peptide (data not shown); therefore cysteines were not alkylated in this method.

We processed magnetic beads with a Magnetic Stand-96 (Life Technologies). Magnetic bead conjugates were added to the digests and incubated with agitation (20 °C for 8 h). We washed the beads with PBS (pH 7.4) and eluted the target peptide/IS with 25 mmol/L glycine, pH 2 (75 μL). The elution was transferred into a 96-well plate and injected (40 μL) on 2D LC-MS/MS. The overall time required for sample preparation in a 96-well plate format was approximately 20 h.

LC-MS/MS ANALYSIS

Two-dimensional HPLC separation was performed at 30 °C with an HPLC system consisting of series 1260 and 1290 pumps (Agilent Technologies). We used a Zorbax XDB-CN 50x2.1, 5-μm HPLC column (Agilent Technologies) for the first-dimension separation, with gradient of mobile phases 98% A to 87% A in 1.3 min (A, 10 mmol/L FA in water; B, 10 mmol/L FA in

acetonitrile); for the second-dimension separation, we used a Poroshell 120EC-C18, 100x3, 2.7-μm column (Agilent Technologies) and a gradient of the same mobile phases 87% A to 75% A in 2 min.

We performed quantitative analysis using an API 5500 triple-quadrupole mass spectrometer with a V-spray ionization source operated in positive-ion multiple reaction monitoring mode. Mass transitions monitored in the method were *m/z* 636.36/1059.56, 636.86/1060.56, 636.36/912.49, and 636.36/541.35 for the VIFDANAPVAVR peptide and *m/z* 639.34/1065.56, 639.84/1066.56, 639.34/918.48, and 639.34/547.34 for the IS. We adjusted the instrument settings to maximize analytical sensitivity and specificity of detection. Relevant instrument parameters are provided in the online Supplemental Materials. Total analysis time per sample was 6.5 min. Data were processed with software (Analyst 1.5.2).

We measured total protein concentration using a spectrophotometric method with a NanoDrop™ 8000 (Thermo Scientific) and concentrations of IgG and albumin using an automated immunoassay analyzer (BN II, Dade Behring) and Modular Analytics (Roche Diagnostics), respectively. We performed the Tg-AAb test on an automated immunoassay analyzer (Immulite 2000, Siemens).

METHOD VALIDATION

Method validation consisted of evaluation of imprecision, lower LOQ, linearity, comparison of the method with commercial immunoassay and a LC-MS/MS method of another laboratory, recovery, carryover, ion suppression, and the establishment of reference intervals for Tg. Precision experiments used pools of human serum samples supplemented with Tg (AbD Serotec). We determined LOQs as the lowest concentrations at which values were within ±15% of target, imprecision was <15%, and the ratio of mass transitions was maintained within ±30%. We determined the limits of detection (LODs) as the lowest concentrations at which chromatographic peaks were present in all mass transitions and signal-to-noise ratio was >5. We evaluated the linearity of the method by analyzing 7 samples prepared by mixing different proportions of 2 serum pools containing Tg at 5 and 1045 ng/mL (3.3 and 886 pmol/L).

We compared the method with the Access™ Beckman Coulter DxI800 Tg IA performed at ARUP Laboratories. Samples included those that tested negative for Tg-AAb (n = 73) and positive for Tg-AAb (n = 113). Samples with concentrations of Tg-AAb <20 IU/mL were considered Tg-AAb negative. In the Tg-AAb–positive samples, the concentrations of Tg-AAb were 20–3000 IU/mL and Tg recovery was 5%–97%. We used the ratio of the concentrations determined from multiple mass transitions to evaluate the specificity of analysis *(15)*.

We determined magnetic bead enrichment recovery by performing affinity enrichment of a pool of digested serum samples containing 80 ng/mL Tg. Linearity of sample dilution (with Tg-negative serum) was evaluated by analyzing a serum sample containing >3000 ng/mL Tg (measured with LC-MS/MS).

We evaluated the effects of lipemia, hemolysis, and icterus by analyzing pools of normal serum and lipemic, hemolysed, and icteric samples as is and mixed 1:1; we compared the observed concentrations with concentrations measured in the individual samples. Ion suppression was evaluated with the post–column infusion method *(16)*. We performed tube-type studies with blood collected in potassium EDTA, sodium heparin, serum, and serum separator tubes. Storage stability of Tg was evaluated at room temperature, 4 °C, and −20 °C after 1, 2, 3, 4, 8, 14, 21, and 28 days of storage.

We performed the reference interval study for Tg with samples from self-reported healthy adult volunteers (25 men and 25 women) and 140 samples from children ages 1, 4, 7, 10, 13, and 16 years (10 samples from each sex of each age). All studies with human samples were approved by the Institutional Review Board of the University of Utah.

Differences in concentrations between the groups in the experiments for evaluation of different types of collection tubes and problem sample types were evaluated with ANOVA.

Results

Mass transitions were selected from product ion mass spectra of the Tg-specific peptide (VIFDANAPVAVR) and the IS peptide (Fig. 1 and online Supplemental Fig. 1). Free and AAb-bound Tgs were enriched from serum samples with affinity-assisted enrichment (AAE), which consisted of the addition of an anti-Tg antibody to the samples, incubation, and precipitation of the Tg-Ab and Tg-AAb complexes with saturated ammonium sulfate. The supernatants (not containing Tg) were discarded, and the precipitates containing Tg were further processed. We evaluated the effects of the concentration of the rabbit antibody and the amount of ammonium sulfate on distribution of Tg, total protein, albumin, and IgG content in the supernatants and precipitates. Online Supplemental Figs. 2 and 3 demonstrate the distribution of Tg, total protein, IgG, and albumin between the supernatants and the precipitates.

Results of the evaluation of assay imprecision are summarized in Table 1. Within-assay imprecision at the evaluated concentrations was <10%, and impreci-

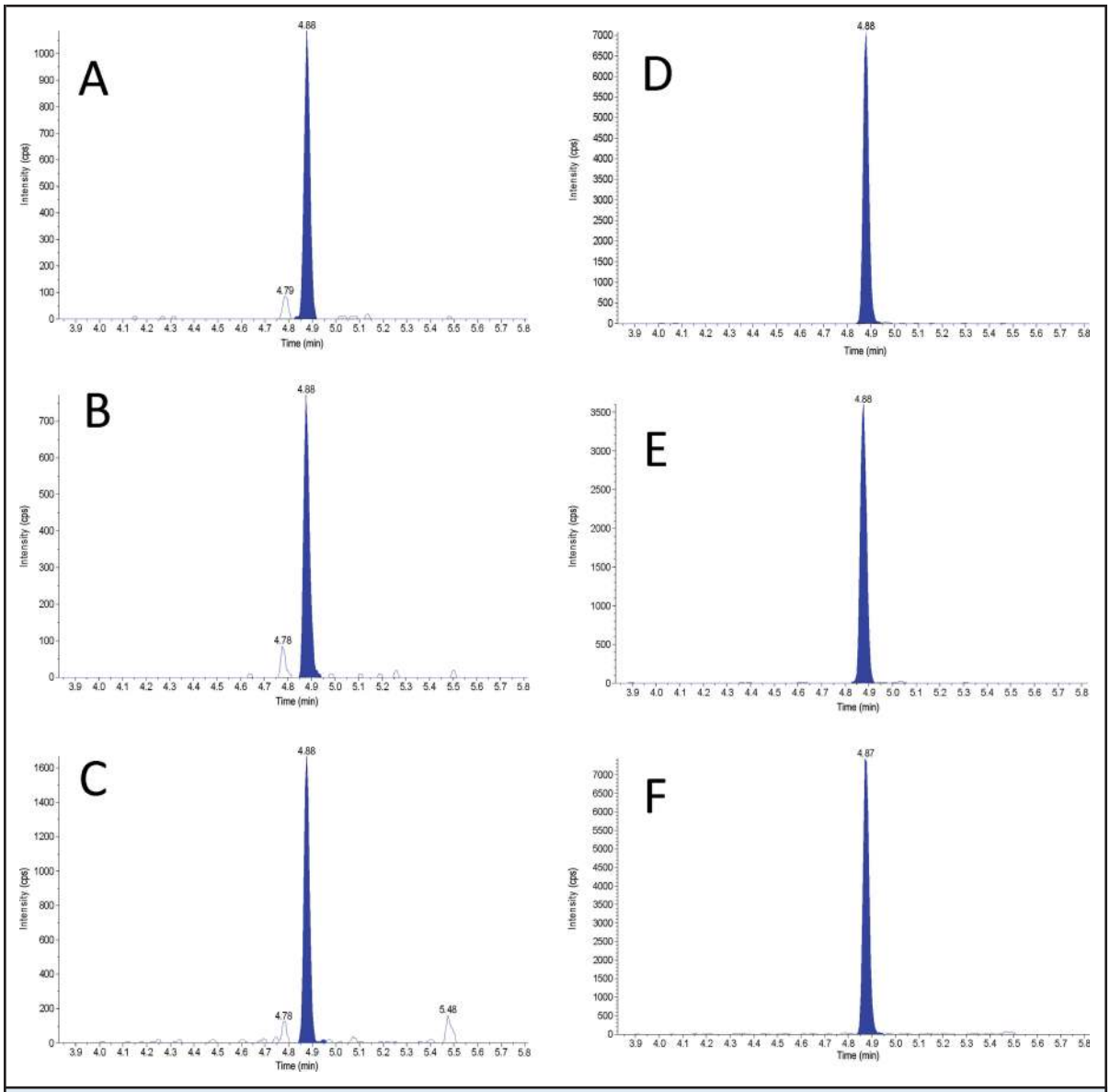

Fig. 1. Chromatogram of patient sample containing 5 ng/mL Tg.

Mass transitions of the VIFDANAPVAVR peptide, *m/z* 636.36/1059.56 (A), 636.36/912.49 (B), and 636.36/541.35 (C), and the IS, *m/z* 639.34/1065.56 (D), 639.34/918.48 (E), and 639.34/547.34 (F).

sion of Tg measurements in QC samples analyzed over 20 days was <14%. The LOQ and LOD of the method were 0.5 and 0.25 ng/mL (0.76 and 0.38 fmol/mL of the Tg dimer), respectively. The method was linear up to 1045 pg/mL, with inaccuracy <10% at the highest concentration. No analyte was detected in an injection of a blank sample immediately after a standard containing 1045 ng/mL (1.58 pmol/mL) Tg. Recovery of the immunoaffinity extraction of the VIFDANAPVAVR peptide was 52.5%. Concentrations observed in the samples from the dilution integrity experiment agreed with each other within 6.8%. Evaluation of Tg stability indicated degradation over 28 days by 44%, 22%, and 13% when stored in polypropylene tubes at room temperature, 4 °C, and −20 °C, respectively (*P* values 0.006 and 0.545 for the difference in slopes of linear regression lines for RT vs 4 °C and 4 °C vs −20 °C, respectively). Three freeze-thaw cycles did not affect the concentration of Tg (see online Supplemental Fig. 4). Considering these data, samples should be analyzed within a

Table 1. Intra-assay, interassay, and total imprecision of LC-MS/MS method for Tg.

Sample	Concentration, ng/mL	Intra-assay, %	Interassay, %	Total, %
Low 1[a]	2.1	6.75	3.67	7.69
Low QC 1[b]	2.3	NA[c]	13.9	NA
Low 2[a]	5.7	6.87	5.96	9.10
Medium QC 2[b]	6.5	NA	10.5	NA
Medium[a]	14.8	6.56	5.40	8.50
High[a]	399	3.56	1.71	3.95
High QC[b]	172.8	NA	3.5	NA

[a] Samples analyzed in 3 replicates per day over 5 days.
[b] Samples analyzed in 1 replicate per day over 20 days.
[c] NA, not applicable.

month after collection if stored frozen at −20 °C and 3 days if stored refrigerated. Analysis of samples collected in 4 different types of collection tubes did not show statistically significant differences in the concentration within an individual (n = 5) (see online Supplemental Fig. 5). Tg concentrations in lipemic, hemolysed, and icteric samples mixed in ratios 1:1 with a normal serum pool were measured as 50% of the total value observed in the individual samples used for preparing the mixtures (see online Supplemental Table 1). This suggests that lipemia, hemolysis, and icterus did not affect performance of this method. A ratio of qualifier to quantifier ions outside of ±30% or the presence of split peaks was considered evidence of the presence of interferences. Among the analyzed (approximately 3000) patient samples, 4.8% of samples had ratios of the mass transitions outside of the ±30% range; Tg concentrations in these samples were 0.5–4 ng/mL. In approximately 20% of these samples, split peaks were observed on chromatograms. Ratio of the mass transitions was not evaluated in samples with concentrations <0.5 ng/mL Tg.

For method comparison, we used the Beckman Access immunoassay analyzer. Deming regression equations for the method comparison with Tg-AAb–negative samples (n = 73) were IA = 1.00 * LC-MS/MS − 2.3, r = 0.982, standard error of the estimate ($S_{y|x}$) = 9.5 (concentrations 0–350 ng/mL) and IA = 0.94 * LC-MS/MS −3.7, r = 0.946, $S_{y|x}$ = 4.2 (concentrations <60 ng/mL, n = 56) (Fig. 2). A set of 113 Tg-AAbs was tested by both methods; concentrations of Tg measured by LC-MS/MS were higher than values determined by IA (Fig. 2C), IA = 0.53 * LC-MS/MS −0.1, r = 0.586, $S_{y|x}$ = 1.1. The disagreement was especially severe at concentrations of Tg <2 ng/mL. Among 71 Tg-AAb–positive samples with concentrations <0.5 ng/mL by Access analyzer, the concentrations in 16 samples (23%) determined by LC-MS/MS method were ≥0.5 ng/mL (median 1.2 ng/mL, range 0.7–11 ng/mL). In the Tg-AAb–positive samples, there was no association between Tg-AAb concentration and Tg concentration determined with LC-MS/MS (see online Supplemental Fig. 6).

We also compared our LC-MS/MS method with an LC-MS/MS method at the University of Washington *(12)* using a set of Tg-AAb–negative (n = 21) and Tg-AAb–positive (n = 29) samples (Fig. 3). For Tg-AAb–negative samples, LC-MS/MS_{UW} = 1.17 * LC-MS/$MS_{eval.}$ − 1.81, r = 0.951, $S_{y|x}$ = 8.14; for the Tg-AAb–positive samples, LC-MS/MS_{UW} = 1.23 * LC-MS/$MS_{eval.}$ + 0.15, r = 0.917, $S_{y|x}$ = 0.475. Poor agreement at concentrations <2 ng/mL of Tg was observed between concentrations determined with LC-MS/MS assays and the Beckman Access immunoassay.

Reference intervals of Tg established with our method were 5.7–45.5 ng/mL (8.6–68.9 pmol/L) in 1- to 4-year-olds; 1.4–36.3 ng/mL (2.1–55 pmol/L) in 7- to 17-year-olds; and 1.1–36.7 ng/mL (1.7–55.6 pmol/L) in 18-year-olds and older individuals.

Discussion

We developed a method for measuring Tg that overcame interference of Tg-AAbs. The method was based on the enrichment of all forms of Tg (free and AAb bound), followed by proteolytic digestion, IAE, and 2D LC-MS/MS analysis of the Tg-specific peptide. The assay consisted of the following steps: (*a*) addition to serum samples of anti-Tg Ab; (*b*) enrichment of Tg through precipitation of Tg-Ab and Tg-AAb complexes; (*c*) redissolving the precipitate with solvent containing IS; (*d*) denaturing, reduction, and proteolytic digestion; (*e*) enrichment of the targeted peptide with antipeptide antibody conjugated to magnetic beads; (*f*) removal of nonspecifically bound peptides by washing the beads; (*g*) elution of the targeted peptide; and (*h*) LC-MS/MS analysis.

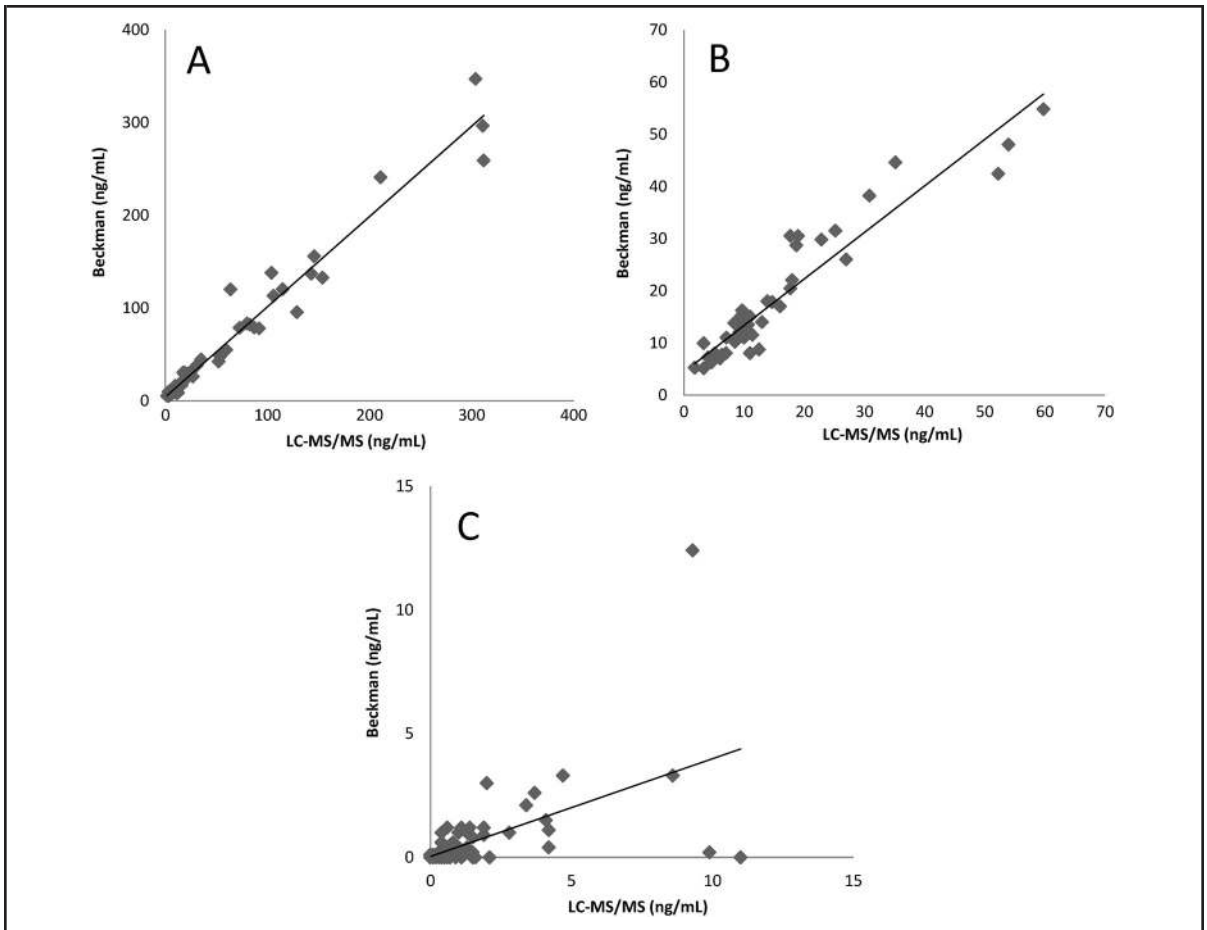

Fig. 2. LC-MS/MS method comparison with Access analyzer (Beckman Coulter) with Tg-AAb–negative (A, B) and Tg-AAb–positive (C) samples.

(A), Concentrations 0–350 ng/mL, IA = 0.99 * LC-MS/MS − 3.1, $r = 0.980$, $S_{y|x} = 14.8$. (B), Concentrations <60 ng/mL, IA = 0.94 * LC-MS/MS − 3.7, $r = 0.946$, $S_{y|x} = 4.2$. (C), IA = 0.53 * LC-MS/MS − 0.1, $r = 0.586$, $S_{y|x} = 1.1$.

Many patients have Tg-AAbs that can interfere with the quantification of Tg by immunoassays. Gentile et al. *(17)* identified 19 epitopes on the Tg molecule, which could participate in autoantibody binding. In samples from patients treated for TC, there is a need for accurate measurement of total concentrations of Tg (free and AAb bound) as a means of detecting the recurrence of TC. Interindividual differences in the epitopes and the affinity of Tg-AAb toward Tg make it impossible to reliably and accurately measure Tg with immunoassays in every sample containing AAbs *(9)*.

The novel AAE strategy used in this method relies on the conversion of all Tg in biological samples to an Ab-bound (and AAb-bound) form, followed by precipitation of the Tg-antibody complexes with ammonium sulfate. A large fraction of nontargeted blood proteins are simultaneously depleted (approximately 70%) during the enrichment step. Partial enrichment of Tg was also observed in samples not containing Tg-AAb (see online Supplemental Fig. 3). This likely occurred through coprecipitation of Tg nonspecifically bound to Ig. In samples that did not contain Ig, Tg did not precipitate with ammonium sulfate and remained in solution (data not shown). Addition of polyclonal anti-Tg antibody to patient samples enhanced the efficiency of enrichment, likely owing to antibody binding to free Tg, which resulted in the enhanced partitioning of the antibody-Tg complexes to the Ig-containing fraction. The AAE therefore allowed the precipitation of free and AAb-bound Tg in a single fraction, while reducing (*a*) sample complexity before the digestion, (*b*) amount of trypsin used, and (*c*) complexity of the resulting tryptic digests.

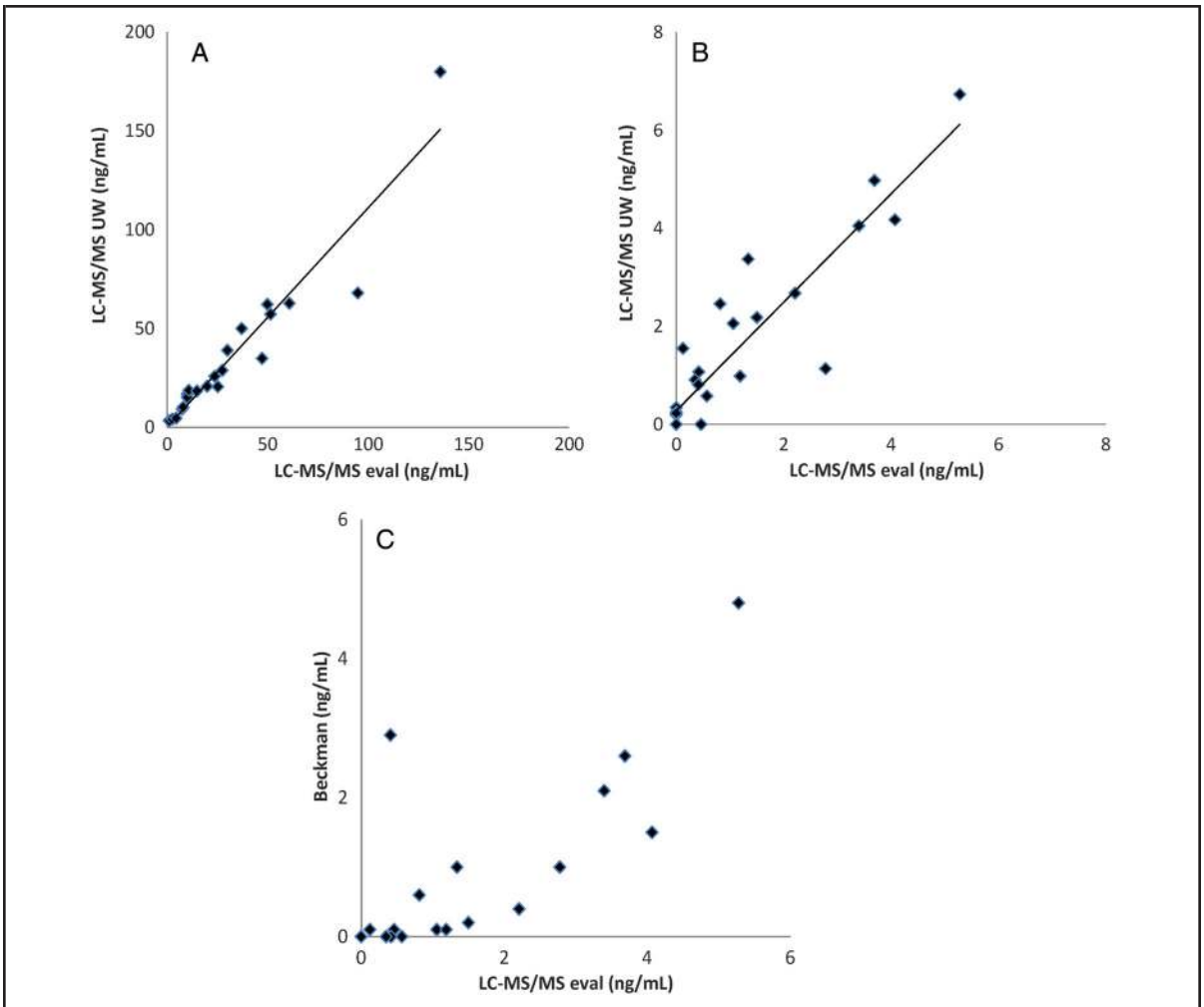

Fig. 3. Comparison of the developed LC-MS/MS method with an LC-MS/MS method at the University of Washington *(12)* using Tg-AAb–negative (n = 21) (A) and Tg-AAb–positive (B) (n = 29) samples, and (C), comparison with Access analyzer (Beckman Coulter) with Tg-AAb–positive samples (samples corresponding to the results shown in (B).

(A), LC-MS/MS$_{UW}$ = 1.17 * LC-MS/MS$_{eval.}$ − 1.81, r = 0.951, $S_{y|x}$ = 8.14. (B), LC-MS/MS$_{UW}$ = 1.23 * LC-MS/MS$_{eval.}$ + 0.15, r = 0.917, $S_{y|x}$ = 0.475. UW, University of Washington; eval, evaluation.

Multidimensional separations are widely used in the analysis of small molecules from complex matrices *(18–21)*. By the same concept, we optimized an online 2D separation for the targeted peptide using 2 chromatographic columns with complementary retention mechanisms under ultraperformance liquid chromatography conditions. Optimal selectivity was achieved by combining the first-dimension column having weak retention with a second-dimension column that strongly retained the peptide. We optimized the separation conditions to provide a good separation from other endogenous species while maintaining a short analysis time. Although the instrumentation and the method with multidimensional chromatography are more complex, 2D separations have a number of advantages over separation with a single analytical column. First, in a well-developed method, orthogonal mechanisms of chromatographic separation on the first and second columns could remove peaks that would remain unresolved with a single retention mechanism. Second, peaks eluting outside of the window of the peak transfer from the first to the second column are directed to waste. Third, there is reduced potential for ion suppression. Fourth, the analytical column has a longer life, and consequently the method is more robust. Last, there is reduced analysis time (reconditioning of the first column takes place while separation takes place on the second column, and vice versa) *(19–21)*.

Digestion of Tg with trypsin produces >400 peptides, among which 3 were suggested as suitable for quantitative measurements of Tg *(11, 12)*. In our method, the peptide VIFDANAPVAVR was used as a surrogate marker for quantification of Tg. Advantages of the use of VIFDANAPVAVR peptide for quantitative measurement of Tg are the quantitative yield of the peptide from tryptic digestion and the absence in its sequence of amino acids that could be posttranslationally modified. One of the difficulties of bottom-up proteomics approaches in the quantification of endogenous proteins in human serum lies in the extreme variability of trypsin digestion between experiments *(22, 23)*. In an attempt to minimize this variability, we used a novel approach with a winged peptide, a stable isotope–labeled analog of the VIFDANAPVAVR peptide synthesized with 6 amino acids of the sequence of Tg concatenated at each end. The use of a structurally matched IS containing amino acids beyond the tryptic digestion sites led to improved assay imprecision (data not shown), likely because the IS underwent tryptic digestion in a fashion similar to that of the intact Tg in patient samples. Although the winged peptide-IS does not provide the full spectrum of benefits embodied by a fully stable isotope–labeled protein-analog IS, it does compensate for digestion variability to some extent. Evaluation of the kinetics of trypsin digestion of Tg and the winged peptide showed some differences in the rate of release of the targeted peptide and the stable isotope–labeled peptide analog (data not shown). A similar approach, with an extended sequence peptide-IS, was recently used by Zhang et al. *(24)*. The IS peptide in that method contained an extra amino acid within the sequence of the targeted peptide *(24)*; it is unclear how that modification would affect antibody binding to the IS during the IAE step.

No significant differences were found in distribution of concentrations of Tg between males and females within the same age groups; because of this, we established single reference intervals (nonparametric, central 95% of the distribution) within the age groups in both sexes. Distribution of concentrations of Tg in children and adults is shown in Fig. 4. A statistically significant difference was observed between distributions of concentration in children of age groups 1–4 and 7–17 years. The observed distributions of concentrations and age-dependent differences agree with the reference intervals established with the Beckman Access analyzer *(25)*, supporting the agreement between the LC-MS/MS method and the immunoassay in samples negative for Tg-AAb.

Good agreement was observed between 2 comparative LC-MS/MS methods for both Tg-AAb–negative and Tg-AAb–positive samples. Poor agreement between the LC-MS/MS methods and the Beckman Access immunoassay was observed at concentrations of Tg <2 ng/mL, suggesting that presence of Tg-AAb predominantly affects measurements of Tg in Tg-AAb–positive samples containing <2 ng/mL Tg. Future studies in which clinical information on the participants will be available will be needed for assessment of the association of the recurrence of TC with Tg concentrations determined by LC-MS/MS methods and immunoassays.

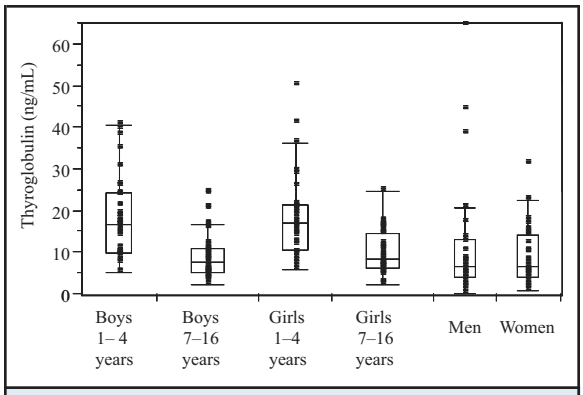

Fig. 4. Distribution of concentrations of Tg in healthy children and adults.

In summary, we developed an IAE 2D LC-MS/MS method for the quantitative analysis of Tg in human serum and plasma in the presence of endogenous Tg-AAbs. The AAE strategy developed here should be generally applicable to other biomarkers for which autoantibodies can be present in clinical samples. The use of 2D LC-MS/MS reduced the complexity of chromatograms and enhanced the LOQ and specificity of the assay. In samples free of Tg-AAbs, we observed good agreement between this LC-MS/MS method and the Beckman Access immunoassay; good agreement was also observed with an LC-MS/MS method in another laboratory for both Tg-AAb–negative and Tg-AAb–positive samples. Poor agreement was observed between the LC-MS/MS methods and the Beckman Access immunoassay in Tg-AAb–positive samples, especially at concentrations <2 ng/mL Tg, which was likely caused by interference of Tg-AAbs with the IA. In a set of Tg-AAb–positive samples that tested negative for Tg with IA, concentrations determined by the LC-MS/MS method were ≥0.5 ng/mL in 23% of samples, which could fundamentally alter the approach for monitoring patients treated for differentiated thyroid carcinoma.

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or revising the article for intellectual content; and (c) final approval of the published article.

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