All that Glitters is Not the Gold Standard: Calibration of a Sensitive Protein Cleavage-Isotope Dilution Mass Spectrometry (PC-IDMS) Assay for Thyroglobulin

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Protein cleavage coupled with isotope dilution mass spectrometry (PC-IDMS) has been utilized in clinical settings to provide interference free measurement of thyroglobulin in the presence of autoantibodies[1-3] and heterophilic antibodies;[4] however, PC-IDMS methods reported to date have sensitivities comparable only to radio immunoassays (RIAs, ca 0.5 to 2 ng/mL) that are used for the same purpose. It has been suggested that functional sensitivities of second generation immunochemiluminescence assays (ICMAs, ca 0.05 to 0.1 ng/mL) are better suited for monitoring recurrence of Differentiate Thyroid Cancer (DTC) as basal concentrations less than 0.2 ng/mL (following thyroidectomy) have a negative predictive value of >95%.[5] To that end, a PC-IDMS assay was developed for quantification thyroglobulin in serum with a lower limit of quantification (LLOQ) of 0.2 ng/mL to provide diagnostic utility comparable to ICMAs, but regardless of autoantibody status.

In order to accommodate the high-throughput demand of a reference laboratory, the presented PC-IDMS assay was developed with a sample extraction time of less than 4.5 hours (tube to autosampler), including protein denaturation, tryptic digestion, and immunoaffinity peptide enrichment. Moreover, an LCMS runtime of less than 2 minutes enabled high volume analysis of patient samples – in excess of 2000 samples per day on a single LCMS system deploying an Aria™ Transcend TX4 system (Thermo Finnigan) coupled to an ABSciex® API5500 (Life Technologies Corporation).

To enable sensitive and accurate measurement at an LLOQ of 0.2 ng/mL, standardization of the assay was investigated with multiple sources of thyroglobulin and multiple matrices lacking endogenous human thyroglobulin, including pooled human sera from remissive thyroidectomy patients (<0.1 ng/mL). Given DTC patients represent a large portion of our thyroglobulin testing population; we were able to create pools of such serum samples (de-identified) to create the ideal calibrator matrix – thyroglobulin-negative sera. Using this matrix, four sources of thyroglobulin were evaluated for preparation of calibrators: BCR® Certified Reference Material (BCR457),

Beckman Access® Thyroglobulin Assay Calibrators (Beckman Coulter), human-derived thyroglobulin (Sigma-Aldrich), native thyroglobulin in serum (sera >250 ng/mL).

High concentration stocks (> 250 ng/mL) of each thyroglobulin source was standardized against the FDA-approved Beckman Access® immunoassay. Both the Beckman Access® and BCR® sources expectedly showed agreement between the immunoassay and label claims when measured in replicate (recovery 90 – 110%). Recovery of the the human-derived material and sera pool were not determined as expected concentrations could not be determined *a priori.* Following standardization, each stock material was diluted with thyroglobulin-negative serum to create the calibration series for the PC-IDMS assay. As might be expected, calibration of the PC-IDMS assay with Beckman calibrators provided good agreement in autoantibody-negative patients between the PC-IDMS assay and Beckman Access® immunoassay (deming slope = 1.075, R = 1.075). Interestingly, calibration of the PC-IDMS assay with the BCR457 material resulted in good correlation of patient results with the immunoassay when using freshly prepared calibrators (deming slope = 0.979, R = 0.9671), but poor agreement following storage of the calibrators (deming slope > 1.3). Measurement of the stored BCR457 working calibrators on the immunoassay showed good recovery of expect values (deming slope = 0.988, R = 0.9993). These results suggest that the BCR457 maintains its immunoreactivity when stored in serum, but has a reduced susceptibility to proteolysis. Similar discordance between the immunoreactivity and digestion behavior was also observed with native thyroglobulin (sera pool) calibrators (deming slope = 0.7090, R = 0.9723) and human-derived thyroglobulin (Sigma-Aldrich) calibrators (deming slope = 1.464, R = 0.9778). A more systematic evaluation of the stability of these calibration materials is currently underway that includes monitoring recovery of the signature peptide during enzymatic digestion as a function of storage duration in sera.

Using the Beckman Access® thyroglobulin standards prepared in thyroglobulin-negative serum, we were able to demonstrate accurate quantification in serum samples with and without antithyroglobulin autoantibodies. However, we often observed a small (<50% LLOQ), though significant interference in the calibrator matrix despite having <0.1 ng/mL of thyroglobulin by an immunochemilumesence assay. This matrix interference resulted in a non-linear response in calibrators near the target LLOQ, thereby preventing reliable quantitation at 0.2 ng/mL. In a previously reported study using Tg-negative serum as the calibrator matrix, this same

observation was not made due to the fact that the reported LLOQ was 2-fold higher in the reported assay.[2]

To eliminate the interference from human thyroglobulin and facilitate reliable calibration at 0.2 ng/mL, several animal serum were tested as surrogate matrices. Accurate quantification was obtain in the majority of sera as demonstrated through accurate recovery upon dilution of patient samples with the surrogate matrices, with the exception of chicken and procine serum. Porcine serum possessed an unknown interferent despite lacking the signature peptide sequence, while chicken serum resulted in a positive bias (>80%) due to altered digestion behavior that was elucidated through timecourse analysis of the digestion step. Despite accurate recovery in several animal sera, all resulted in a matrix effect that decreased analytical sensitivity by >5-fold.

Finally, the use of an artificial matrix created from human serum albumin diluted into PBS at physiologically relevant concentrations was explored. Comparison of 40 to 80 mg/mL HSA all showed accurate recovery upon dilution of serum (95 to 105%), without impacting analytical sensitivity and with no evidence of interference. Calibration of the PC-IDMS assay with thyroglobulin from Beckman Access® calibrators diluted into the artificial matrix has demonstrated good correlation with Beckman Access® immunoassay in autoantibody negative patients (deming slope = 1.015, R = 0.9816), as well as good agreement between two signature peptides (deming slope = 0.967, R = 0.9841). Moreover, repeatability at 0.2 ng/mL showed acceptable imprecision (11.8%) and recovery (109.2%) with this calibration system. A functional sensitivity study is currently underway to establish the lowest concentration of thyroglobulin in serum that can be measured with an imprecision of less than 20% below the validated LLOQ of 0.2 ng/mL.

References

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