QUANTIFICATION OF THYROGLOBULIN IN SERUM FOR CLINICAL RESEARCH USING SISCAPA WORKFLOW COMBINED WITH LC-MS/MS

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INTRODUCTION

Thyroglobulin (Tg) is a 660 kDa homodimer synthesized by the follicular cells of the thyroid gland, acting as a substrate in the production of the hormones triiodothyronine (T3) and thyroxine (T4). Accurate measurement of Tg using existing immunoassay-based techniques can be challenging in the presence of anti-Tg antibodies (TgAb), which can prevent the binding of Tg to assay antibodies thus leading to non-quantifiable Tg concentrations. The Stable Isotope Standards and Capture with Anti-Peptide Antibodies (SISCAPA) workflow combined with liquid chromatography - tandem mass spectrometry (LC-MS/MS) has been successfully employed to circumvent this problem by digesting the serum sample thus eliminating interfering TgAb and measuring a Tg-specific surrogate peptide instead. Here we report the development of a SISCAPA™ workflow for accurate measurement of thyroglobulin on an Andrew+™ Pipetting Robot, enabling easier adoption of this workflow.

METHODS

Materials

- 10µg/mL thyroglobulin Certified Reference Material (CRM) (Merck, UK) was used to create calibrators in Tg negative surrogate serum matrix (chicken serum) (Merck, UK) over the range of 0.1- 50 ng/mL.
- In-house Quality Controls (QCs) were prepared using pooled human serum (BioIVT, UK) and surrogate serum to create pools at 0.3, 3 and 35 ng/mL.
- FSPDDSAGASALLR*(*$^{13}C_6$, $^{15}N_4$) (Biosynth, USA) was used as the Stable Isotope Labelled (SIL) internal standard.

Sample Digestion and SISCAPA Workflow

- The method was automated using the Andrew+ Pipetting Robot with OneLab™ Software. 250µL serum was manually transferred to a Axygen™ 1.1mL 96-well collection plate prior to placement on the automation.
- 100µL of denaturant and reducing agent was added to each sample. Samples were mixed for 40 minutes at 37°C. 30µL of FSP-SIL internal standard was added to each sample and mixed.
- 50µL of Trypsin was added. Samples were mixed for 30 minutes at 37°C. 25µL cOmplete™ Protease Inhibitor Cocktail(aq) was added to each sample and mixed for 10 minutes.
- 25µL of diluted SISCAPA Tg beads (1 in 2.5 dilution with wash reagent) were added to each sample and the samples were mixed for a total of 60 minutes.
- The collection plate was added to a magnetic array, allowing the beads to bed pull down and washed twice with 250µL wash reagent.
- 50µL of elution buffer was added to each sample. The collection plate was mixed, and the plate was transferred back to the magnetic array to allow elution transfer to a 700µL 96-well collection plate. The collection plate was sealed and an autosampler magnetic plate was secured to the collection prior to injection.

LC-MS/MS Parameters

- Injection was performed in partial loop mode using an ACQUITY™ UPLC™ I-Class FL System. Thyroglobulin were separated using a XSelect™ Premier HSS T3 Column, 100Å, 2.5 µm, 2.1 X 50 mm, with mobile phases of 0.01% formic acid(aq) and acetonitrile, over a run time of 2.6 minutes.
- Detection was performed using a Xevo™ TQ Absolute Mass Spectrometer, with MRM transitions targeting the thyroglobulin FSP peptide at 704.0 > 586.9 and 704.0 > 687.4. The FSP SIL internal standard was monitored at 709.0 > 591.9.

RESULTS

Analytical Sensitivity

- Functional sensitivity tests (three runs, n=30 at each level) demonstrate precision of <20% with S/N > 10:1 for Tg at 0.1 ng/mL.
- Low-level thyroglobulin in human serum demonstrates excellent analytical sensitivity for this method (Figure 1).

THYROGLOBULIN CAN BE QUANTIFIED AT 0.1ng/mL FROM 250µL SERUM USING SISCAPA WORKFLOW, CONVENTIONAL FLOW CHROMATOGRAPHY AND MS/MS
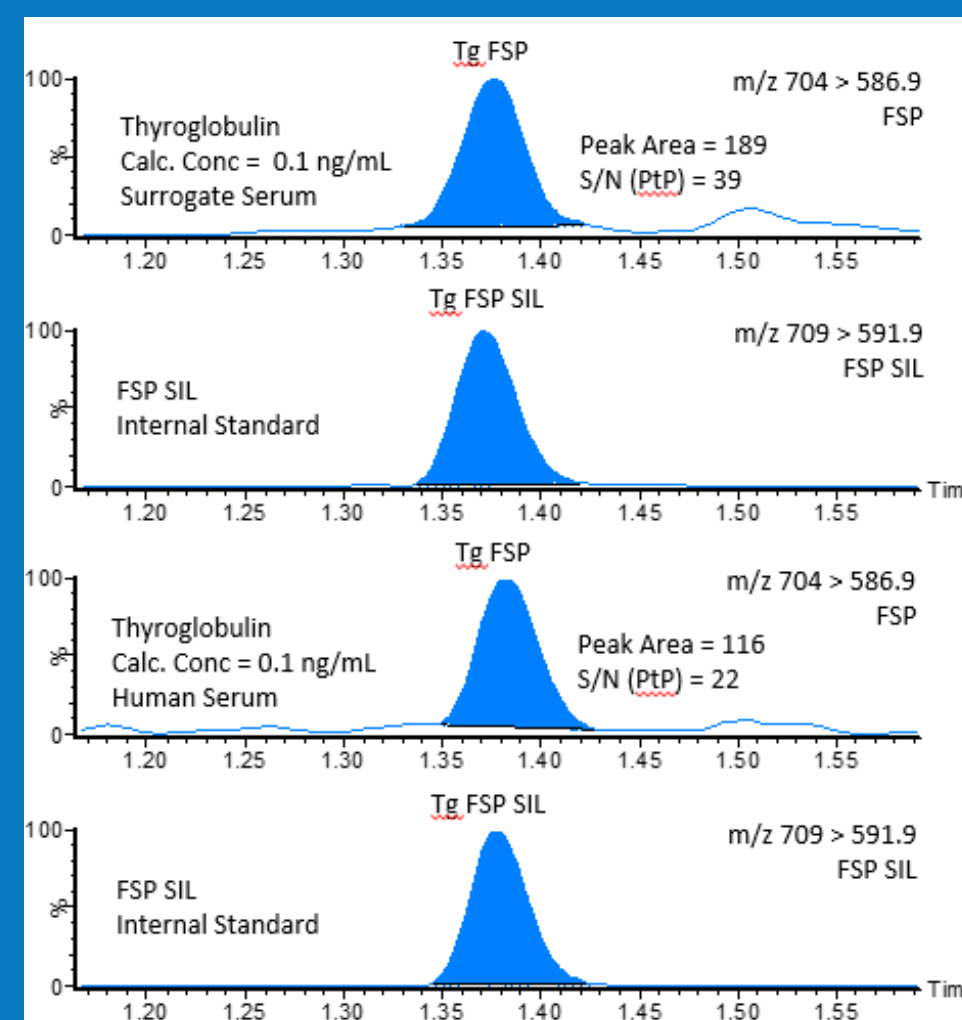

Figure 1. Thyroglobulin quantified at 0.1ng/mL in both surrogate serum and human serum

Link to Application Note

QC Precision

- Reproducibility and repeatability were determined by extracting and quantifying five replicates of three level QC material per day over five analytical runs (n=25).
- Reproducibility and repeatability for human serum/surrogate serum QCs at 0.3, 3 and 35ng/mL for thyroglobulin were ≤9.5% CV for both manual and automated sample preparation.

Matrix Effects

- Matrix effects were evaluated by extracting six individual human serum samples in triplicate and post-spiking samples with a high concentration of FSP peptide and FSP SIL peptide. Endogenous thyroglobulin concentrations were compensated for through extraction of additional blank samples.
- Matrix Effects based on peak area ranged from 79% to 104%, with <9% RSD. Matrix effects based on analyte: internal standard response ratio ranged from 96% to 105%, with <4% RSD, demonstrating the internal standard compensates for changes in matrix effects observed for the method.
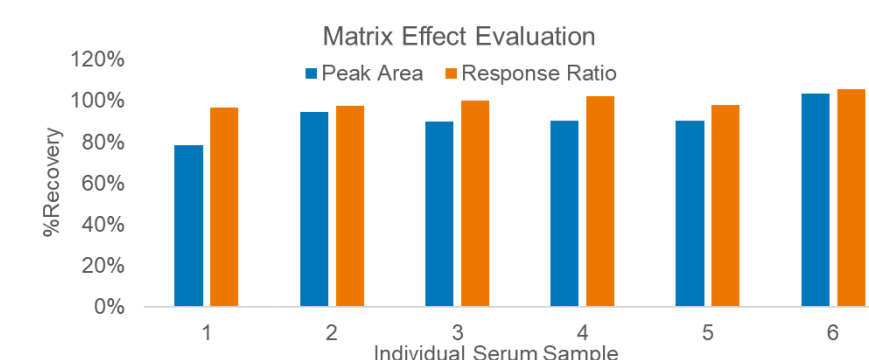

Figure 2. Calculated matrix effects based on both Tg FSP peak area and FSP:FSP SIL response ratio across six individual human serum samples

EQA and Method Comparison

- 38 samples from UK NEQAS were evaluated and compared to the method All Laboratory Trimmed Mean (ALTM).
- Passing-Bablok analysis demonstrates significant proportional bias with a regression of y=0.2149 + 0.5861x. This is further verified by the Bland-Altman agreement which demonstrates a -40.1% method bias of the developed method against the ALTM, indicating possible differences between method calibration.
- This was confirmed through reassignment of the in-house calibrators using the EQA ALTM, leading to a reduction in method bias to -5.5%, and a Passing-Bablok regression of y=-0.261 + 0.9384x (Figure 3), indicating differences in the metrological traceability of the calibration material.
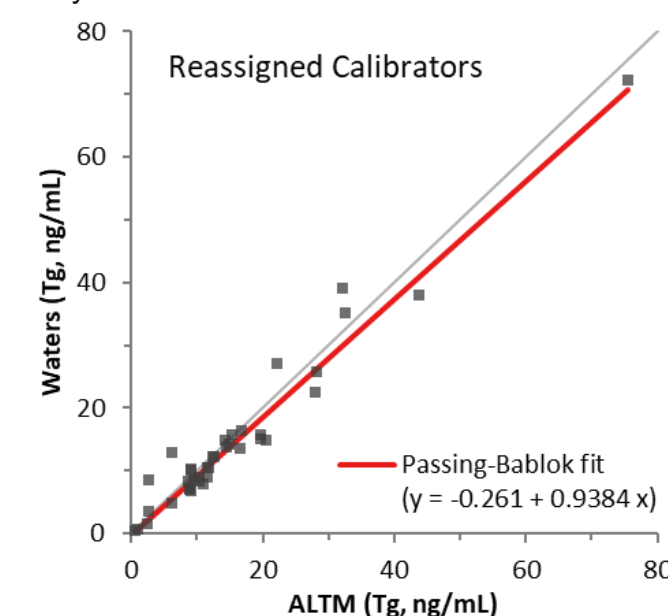

Figure 3. Passing-Bablok analysis of 38 Tg EQA samples analyzed using the Waters developed method after calibrator re-assignment

CONCLUSION

- **A LC-MS/MS clinical research method for serum thyroglobulin was developed using SISCAPA, followed by analysis using ACQUITY UPLC I-Class PLUS FL System and the Xevo TQ Absolute Mass Spectrometer.**
- **The method provides analytical sensitivity down to 0.1ng/mL from 250µL serum, while providing sufficient sample for re-analysis.**
- **Total reproducibility and repeatability of the method was ≤9.5% CV, with EQA bias of –5.5% (following reassignment) using the Andrew+ Pipetting Robot.**