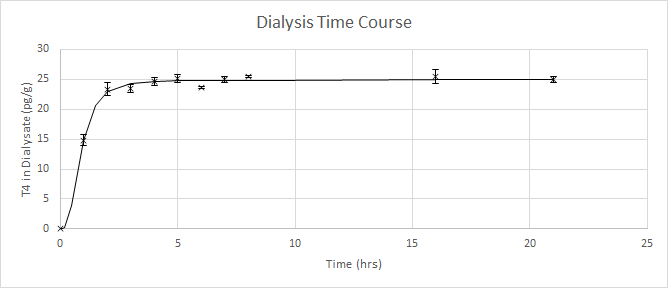
Supplementary material

**Development of Equilibrium Dialysis ID-UPLC/MS/MS Candidate Reference Measurement Procedure for Free Thyroxine in Human Serum**

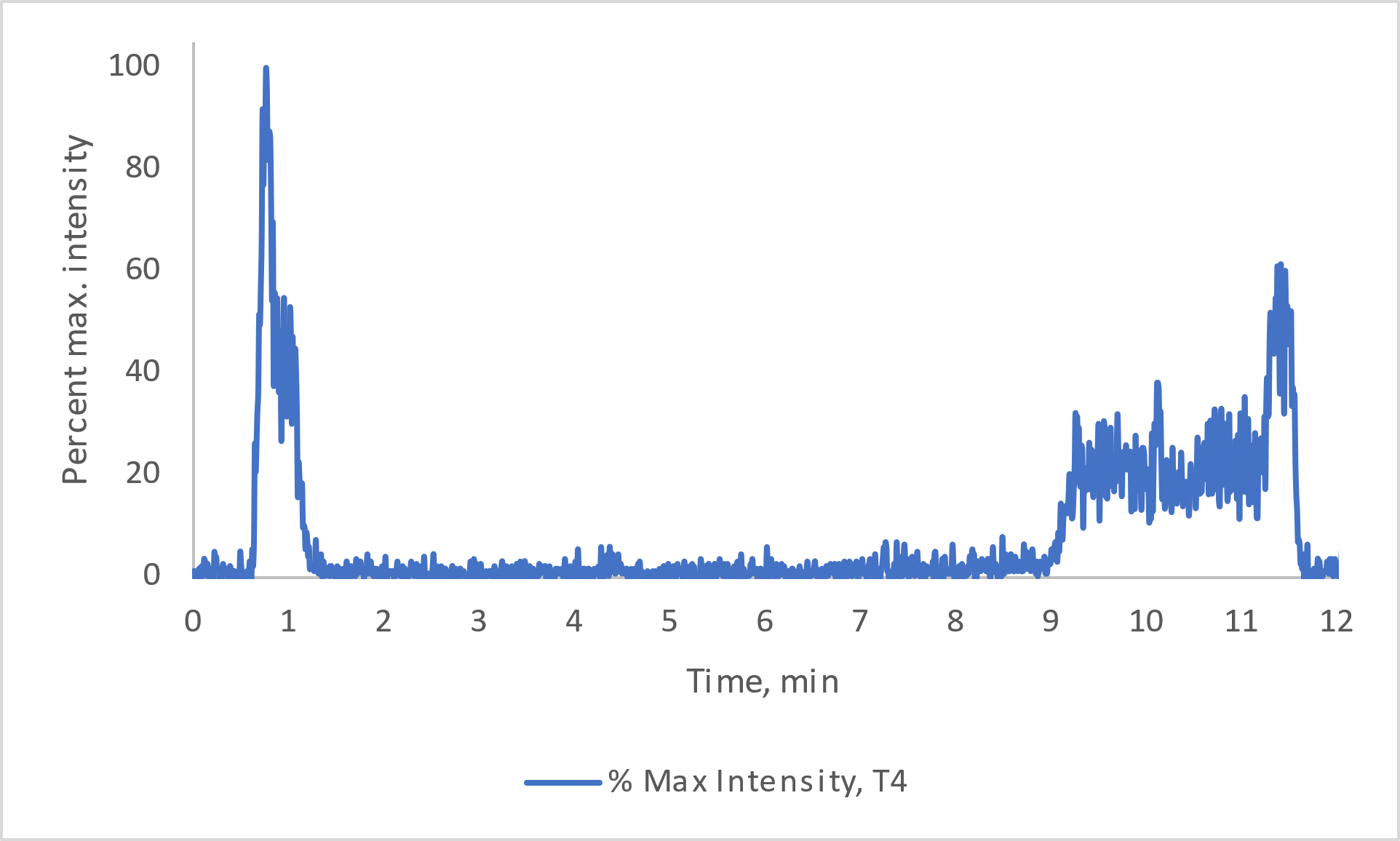
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Supplementary Figure S1. FT4 concentrations obtained at different equilibrium durations (mean concentration +/-1SD) using method A.

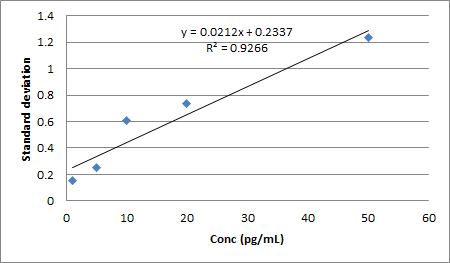
The FT4 concentration at 1-hour intervals from 0–8 hours and at 16 and 21 hours was measured in duplicate. A plateau in concentration is observed at 4 hours, indicating that equilibrium is reached.



B

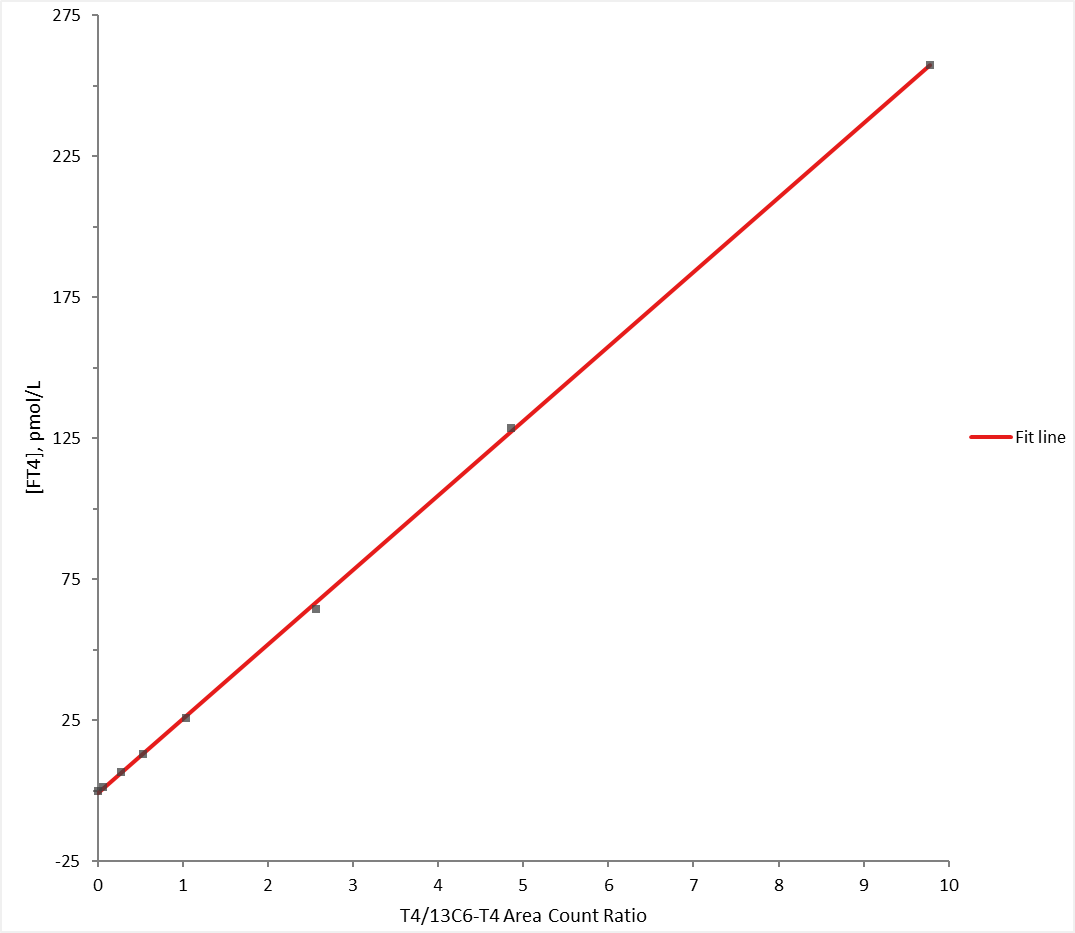
A

Supplementary Figure S2. Representative total ion chromatogram of (A) a blank sample HEPES dialysis buffer extracted by method A and (B) a neat blank containing 38.1 pmol/L 13C6-T4. No interferences were detected around the retention time of T4 and 13C6-T4 (7.0 minutes).



Supplementary Figure S3. Estimation of LOD

To determine the LOD, 5 levels of T4 spiked in HEPES dialysis buffer (1–50 pg/mL) were analyzed and plotted against the standard deviation of replicate measurements of each level measured in duplicate over 3 days. The LOD was estimated as 3 times the standard deviation at the extrapolated concentration of 0.



|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Analyte | Model | Linear Model Residuals | Quadratic Model Residuals | Probability |
| FT4 | Not Weighted | 0.015863 | 0.016038 | 0.57263 |
| FT4 | 1/SQRT(x) | 0.000043 | 0.000040 | 0.03118 |
| FT4 | 1/x | 0.000004 | 0.000003 | 0.00017 |
| FT4 | 1/x2 | 0.000000 | 0.000000 | 0.00006 |
| FT4 | 1/y | 0.000007 | 0.000005 | 0.00002 |

Supplementary Figure S4. Linearity assessment

The linear model of a 7-point calibration curve (not weighted) with T4 concentrations at 1.29, 6.44, 12.9, 25.7, 64.4, 128, and 257 pmol/L was shown in the graph, and the R2 value was 1.000 supporting excellent fit. The residuals of linear and nonlinear models (not weighted) were comparable. Considering best fitting and model simplicity, a linear model was chosen to construct calibration curves.

|  |  |
| --- | --- |
| Sample ID | Sample Type |
| RB | Run blank |
| INST | System suitability sample |
| RB | Run blank |
| CC1\_1 | Calibrator level 1 replicate 1 |
| CC2\_1 | Calibrator level 2 replicate 1 |
| CC3\_1 | Calibrator level 3 replicate 1 |
| CC4\_1 | Calibrator level 4 replicate 1 |
| CC5\_1 | Calibrator level 5 replicate 1 |
| ISBlank\_1 | Internal standard blank replicate 1 |
| RB | Run blank |
| QC1\_1 | QC sample 1 replicate 1 |
| QC1\_2 | QC sample 1 replicate 2 |
| QC1\_3 | QC sample 1 replicate 3 |
| QC2\_1 | QC sample 2 replicate 1 |
| QC2\_2 | QC sample 2 replicate 2 |
| QC2\_3 | QC sample 2 replicate 3 |
| Sample1\_1 | Patient sample 1 replicate 1 |
| Sample1\_2 | Patient sample 1 replicate 2 |
| RB | Run blank |
| CC1\_2 | Calibrator level 1 replicate 2 |
| CC2\_2 | Calibrator level 2 replicate 2 |
| CC3\_2 | Calibrator level 3 replicate 2 |
| CC4\_2 | Calibrator level 4 replicate 2 |
| CC5\_2 | Calibrator level 5 replicate 2 |
| ISBlank\_2 | Internal standard blank replicate 2 |
| RB | Run blank |
| Sample2\_1 | Patient sample 2 replicate 1 |
| Sample2\_2 | Patient sample 2 replicate 2 |
| Sample3\_1 | Patient sample 3 replicate 1 |
| Sample3\_2 | Patient sample 3 replicate 2 |
| Sample4\_1 | Patient sample 4 replicate 1 |
| Sample4\_2 | Patient sample 4 replicate 2 |
| Sample5\_1 | Patient sample 5 replicate 1 |
| Sample5\_2 | Patient sample 5 replicate 2 |
| RB | Run blank |
| CC1\_3 | Calibrator level 1 replicate 3 |
| CC2\_3 | Calibrator level 2 replicate 3 |
| CC3\_3 | Calibrator level 3 replicate 3 |
| CC4\_3 | Calibrator level 4 replicate 3 |
| CC5\_3 | Calibrator level 5 replicate 3 |
| ISBlank\_3 | Internal standard blank replicate 3 |
| RB | Run blank |
| INST | System suitability sample |
| RB | Run blank |

Supplementary Table S1. Structure of the Analytical Series

Calibrators and serum materials are alternated with run blanks during analysis by LC-MS/MS. All samples are typically prepared in duplicate, while the 2 levels of quality control (QC) materials and 5-point calibration curves are prepared in triplicate. Samples, QC, and calibrators are analyzed in triplicate in a 24-hour period by repeat injections to minimize variability related to injection and analysis. Repeat measurements were performed on different days during value assignment.

|  |  |  |  |
| --- | --- | --- | --- |
| **Analogs of Thyroxine** | **MW (g/mol)** | **Retention**  **Times (min)** | **Vendor** |
|
| 3,5,3'-triiodothyronine | 650.97 | 6.62 | Sigma-Aldrich, St. Louis, MO |
|
| 3,3',5'-triiodothyronine | 650.97 | 6.16 | Sigma-Aldrich, St. Louis, MO |
|
| 3,5-Diiodothyronine | 525.08 | 5.04 | Santa Cruz Biotechnology, Inc., Dallas, TX |
| 3,3’-Diiodothyronine | 525.08 | 5.84 | Santa Cruz Biotechnology, Inc., Dallas, TX |
| 3-Iodothyronine | 399 | 4.34 | Santa Cruz Biotechnology, Inc., Dallas, TX |
| Thyronine | 273 | 3.04 | Santa Cruz Biotechnology, Inc., Dallas, TX |
| 3-Iodothyronamine | 355 | 4.40 | Sigma-Aldrich, St. Louis, MO |
| 3,5-Diiodo-tyrosine | 433 | 0.69 | Sigma-Aldrich, St. Louis, MO |
| 3-Iodo-tyrosine | 307 | 0.69 | Sigma-Aldrich, St. Louis, MO |
| Tyrosine | 181 | 0.69 | Sigma-Aldrich, St. Louis, MO |
| 3,5,3'-Triiodothyroacetic acid | 621.93 | 7.79 | Sigma-Aldrich, St. Louis, MO |
| 3,3′,5,5′-Tetraiodothyroacetic acid | 747.83 | 8.42 | Sigma-Aldrich, St. Louis, MO |
|
|
|

Supplementary Table S2. List of Compounds Tested for Interference

Structural analogs of T4 were assessed for potential interference to T4 (retention time=6.95 min) by comparing their retention times. None of the 12 compounds tested interfered with T4.

Supplementary

Uncertainty Calculation

Type A budget derives from the imprecision of samples used for uncertainty calculation. The intra-assay, inter-assay, and total percent coefficients of variation (CVs) were determined, according to the principles described in CLSI EP 5-A3.

Type B budget

|  |  |
| --- | --- |
| Type B budget | Contribution to the standard uncertainty budget |
| Purity of the IRMM-468 Thyroxine | 0.35% |
| Analytical balance | negligible |
| Uncertainty associated with the density meter | 0.1% |

utypeB =(upurity 2+udensity measurements2)1/2

usample= (utypeA 2+utypeB2)1/2

Supplementary Table S3. Calculation of measurement uncertainty

Potential sources of uncertainty were evaluated and used to calculate the standard uncertainty and expanded uncertainty, according to ISO Guide to the Expression of Uncertainty in Measurement 2008. The estimated variance of Type A uncertainty was obtained from imprecision of the repeated measurements inclusive independent ED. Type B uncertainty was estimated from uncertainties in the purity of the primary reference material, inaccuracy in the weighing of each component, and the measurement of the serum dialysate density. Type A and B uncertainties were combined quadratically to determine the standard uncertainty. Expanded uncertainty, at the 95% confidence level, was determined by multiplying standard uncertainty by a coverage factor, k=2.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | Calibrator T4 QI/CI | | |  |  | Sample T4 QI/CI | | |
|  |  | Replicate 1 | Replicate 2 | Replicate 3 |  |  | Day 1 | Day 2 | Day 3 |
| Day 1 | CC Level 1 | 4.49 | 4.42 | 4.35 |  | Sample 1 | 4.43 | 4.61 | 4.48 |
| CC Level 2 | 4.58 | 4.26 | 4.44 |  | Sample 2 | 4.37 | 4.63 | 4.65 |
| CC Level 3 | 4.42 | 4.33 | 4.38 |  | Sample 3 | 4.38 | 4.64 | 4.40 |
| CC Level 4 | 4.50 | 4.35 | 4.41 |  | Sample 4 | 4.57 | 4.53 | 4.40 |
| CC Level 5 | 4.30 | 4.51 | 4.58 |  | Sample 5 | 4.42 | 4.48 | 4.62 |
| Day 2 | CC Level 1 | 4.44 | 4.50 | 4.43 |  | Sample 6 | 4.39 | 4.46 | 4.37 |
| CC Level 2 | 4.44 | 4.70 | 4.62 |  | Sample 7 | 4.64 | 4.45 | 4.51 |
| CC Level 3 | 4.51 | 4.43 | 4.41 |  | Sample 8 | 4.65 | 4.42 | 4.32 |
| CC Level 4 | 4.35 | 4.66 | 4.26 |  | Sample 9 | 4.49 | 4.24 | 4.66 |
| CC Level 5 | 4.57 | 4.49 | 4.33 |  | Sample 10 | 4.46 | 4.65 | 4.60 |
| Day 3 | CC Level 1 | 4.48 | 4.33 | 4.47 |  | Sample 11 | 4.47 | 4.55 | 4.32 |
| CC Level 2 | 4.32 | 4.67 | 4.52 |  | Sample 12 | 4.60 | 4.66 | 4.68 |
| CC Level 3 | 4.43 | 4.39 | 4.46 |  | Sample 13 | 4.37 | 4.71 | 4.62 |
| CC Level 4 | 4.49 | 4.40 | 4.31 |  | Sample 14 | 4.42 | 4.48 | 4.43 |
| CC Level 5 | 4.33 | 4.47 | 4.53 |  | Sample 15 | 4.58 | 4.59 | 4.52 |
| Day 4 | CC Level 1 | 4.67 | 4.42 | 4.51 |  | Sample 16 |  | 4.90 | 4.62 |
| CC Level 2 | 4.42 | 4.49 | 4.63 |  | Sample 17 |  | 4.77 | 4.60 |
| CC Level 3 | 4.52 | 4.59 | 4.53 |  | Sample 18 |  | 4.34 | 4.42 |
| CC Level 4 | 4.53 | 4.58 | 4.53 |  | Sample 19 |  | 4.54 | 4.37 |
| CC Level 5 | 4.64 | 4.39 | 4.51 |  | Sample 20 |  | 4.66 | 4.64 |

Supplementary Table S4. Comparison of QI/CI Ratio between (A) calibrators and (B) single donor sera.

Sets of 5-level calibrators were prepared as described in the Methods section in triplicate over 4 days, and the ratio of the T4 quantitation ion to the T4 confirmation ion were compared to those of 20 single donor samples prepared in singlicate over 2-3 analytical runs. Mean QI/CI ratio ± 95% CI was 4.47 ± 0.03 for calibrators and 4.52 ± 0.04 for samples, with a mean percent difference of sample to calibrator QI/CI of 1.3%.