SIMPLIFIED EQUILIBRIUM DIALYSIS AND SPE METHOD FOR THE UHPLC-MS/MS ANALYSIS OF FREE T4 AND FREE T3 FOR CLINICAL RESEARCH

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BACKGROUND

The measurement of free thyroxine (FT4) and free triiodothyronine (FT3) is typically performed by immunoassay, however these can be prone to analytical interferences. Currently, the accepted gold standard for the measurement of FT4 and FT3 is equilibrium dialysis (ED) combined with LC-MS/MS, which offers improved specificity compared to immunoassay. However, the ED procedure is often time-consuming and typically requires overnight incubation.

Here, we describe a simple clinical research method to perform ED using a commercially available device followed by sample preparation using solid phase extraction (SPE), and UHPLC-MS/MS analysis using the Waters ACQUITY™ UPLC™ System and Xevo™ TQ Absolute Mass Spectrometer. This has allowed for the complete workflow to be performed within a typical working day.

METHODS

Materials

- T4, T3 and their $^{13}C_6$ labelled internal standards were purchased from Merck Life Science (Dorset, UK).
- Calibrators were prepared in 50 mM HEPES buffer with additional salts added at pH 7.40, over 1-100 pg/mL.
- Tri-level Quality Controls (QCs) were prepared in-house 50 mM HEPES buffer with additional salts added at pH 7.40 and an additional individual serum sample for testing the Equilibrium Dialysis (ED) process (BioIVT, UK).

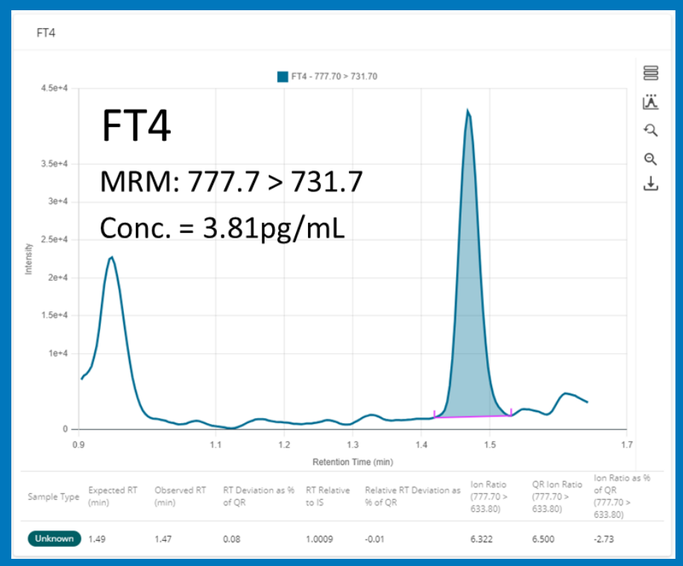
Equilibrium Dialysis and Liquid-Liquid Extraction

- ED was only performed on unadulterated serum samples and the serum QC sample
- 200 µL serum was placed into the sample, chamber of a Rapid Equilibrium Dialysis (RED) insert in a reusable base plate (both Thermo Fisher Scientific, UK)
- 400 µL of dialysate buffer, was added to the buffer chamber device and the plate sealed with sealing tape.
- The plate was mixed in a temperature calibrated orbital shaker for 5 hours at 800 r.p.m. at 37°C
- Post-dialysis, Internal standard and sample diluent were added and samples were transferred onto a pre-conditioned Oasis™ MAX µElution™ Plate
- Samples were washed, eluted and diluted prior to injection on the UHPLC-MS/MS system with no evaporation being required

LC-MS/MS Parameters

- Chromatographic separation was achieved using an ACQUITY PREMIER HSS T3 Column with a water/methanol/formic acid gradient. Total run time was 2.5 minutes (3.5 minutes inj-to-inj).
- FT4 and FT3 and their internal standard were detected in MRM mode on a Waters Xevo TQ Absolute Mass Spectrometer.

Link to Application Note



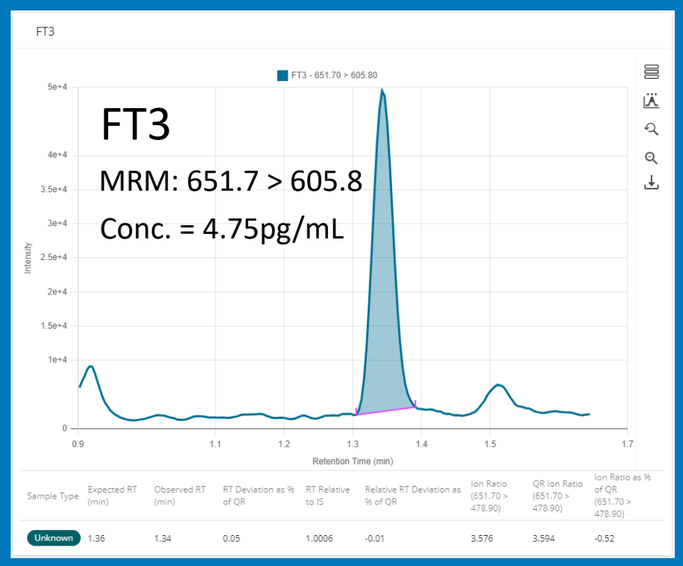


Figure 1. Typical Chromatograms of FT4 and FT3 from human serum samples which have been dialyzed, extracted and analyzed

RESULTS

Chromatography

- Representative chromatograms of unadulterated serum samples can be seen in Figure 1 calculated at the concentrations shown using waters_connect™ with QUAN Review software.

Linearity

- All calibration lines performed during testing had a coefficient of determination (r^2) of >0.995 and %deviations of within ±15 % (±20 % for Cal 1) for both FT4 and FT3 across 1-100 pg/mL.

Precision

- Within-run and total precision were ≤5.0 %CV for the dialysate QC samples (3, 10 & 85 pg/mL) and ≤9.6 %CV for the serum QC sample (FT4: 25 pg/mL, FT3: 6 pg/mL) for both FT4 and FT3.

Analytical Sensitivity and Carryover

- Analytical sensitivity of the method was assessed by extracting and analyzing 10 replicates of dialysate buffer spiked at low concentration levels over 5 occasions. The Lower Limit of the Measuring Interval (LLMI, ≤20%CV precision and ≤15% bias) were determined to be 0.75 pg/mL for both FT4 and FT3.
- No significant carryover was observed from 200 pg/mL samples into subsequent blank samples.

Matrix Factor and Interference Testing

- Matrix effect investigations were evaluated in MSG4000 stripped serum at 2 concentrations and normalized matrix factor calculations, based on the analyte:internal standard response ratio demonstrated mean matrix factors within 0.96-1.10.
- Interference from endogenous compounds (albumin, bilirubin, creatinine, cholesterol, triglycerides & uric acid) were within 85-115 %

Accuracy

- Comparison with CDC HoSt Phase 1 samples (n=40) yielded a Passing Bablok regression of y = 0.9198x + 1.243 (Figure 2) and a Bland Altman bias of 0.070%, indicating strong agreement with the reference method.

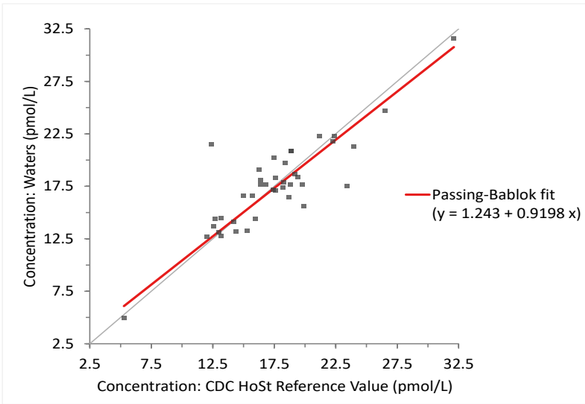


Figure 2: Passing Bablok fit for FT4, comparing our method to the CDC HoSt Reference Measurement Procedure

CONCLUSION

A clinical research method for the analysis of serum FT4 and FT3 has been developed using rapid equilibrium dialysis, simple SPE and fast UHPLC-MS/MS

- **Using only 200 µL of serum, analytical sensitivity of 0.75 pg/mL can be achieved**
- **Excellent reproducibility and repeatability of ≤9.6 % across QC samples**
- **Strong agreement with the CDC HoSt Reference Measurement Procedure has been demonstrated**

The complete workflow can be performed in a standard working day and offers an efficient alternative for thyroid hormone quantification

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