Development of an LC–MS/MS method for the quantification of thyroid hormone precursors Mono-iodotyrosine and Di-diiodotyrosine (MIT and DIT)

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Abstract

Thyroid hormones regulate crucial physiological processes including growth and metabolism and neurodevelopment. Mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) are key intermediates in the thyroid hormone biosynthesis pathway but are rarely quantified due to their low circulating concentrations. The aim of this BMSS/Chom Soc- funded summer studentship is to develop a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of MIT and DIT. Chromatographic conditions, extraction procedures and mass spectrometry parameters were optimised to improve sensitivity, recovery and reproducibility. Solid-phase extraction was used with an Evolute Express AX plate, and chromatographic separation achieved with a Luna Omega Polar C18 column. The developed assay successfully quantified MIT and DIT in mouse thyroid and heart samples, proving its potential as a novel tool for investigating thyroid hormone biosynthesis.

Introduction

Thyroid hormones are essential regulators in human physiology, playing a key role in growth, cellular metabolism, energy homeostasis and neurological development. The thyroid hormone precursors MIT and DIT are key intermediates in thyroid hormone biosynthesis and iodide recycling. The thyroid gland synthesises the biologically active hormones triiodothyronine (T3) and thyroxine (T4) through a tightly regulated series of enzymatic reactions and coupling of the tyrosine residues MIT and DIT. MIT is a tyrosine residue containing one iodine atom and DIT is a tyrosine residue containing two iodine atoms. DIT couples with MIT to make triiodothyroinine (T3) and DIT coupled with another DIT creates thyroxine (T4). Despite their physiological importance, they have been shown to have low circulating concentrations and thus quantifying these analytes comes with many analytical challenges. As a result, current analytical methods focus on T3 and T4, limiting our understanding of thyroid hormone biosynthesis and disorders.(1) This studentship aimed to develop a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for the quantification of MIT and DIT in biological samples as a foundation for future research.(2) This project will enable us to further understand thyroid hormone imbalances and metabolic disorders, providing information on thyroid hormone synthesis, thyroid disorders and defects in pathways.(3)

Materials & Methods

LC-MS/MS is a combination of liquid chromatography and tandem mass spectrometry and is the gold standard in the analysis of metabolic analytes due to its high sensitivity, selectivity and accuracy. Liquid chromatography separates MIT and DIT by polarity (different retention times), and the mass spectrometer measures them by their distinct mass-to-charge ratios (m/z) and fragmentation patterns. Analytical internal standards of stable isotopes $^{13}C_6$ were used throughout method development to improve quantitative accuracy and extraction variability.

Method development was performed using a Waters-S. Optimisation focused firstly on determining suitable ionisation conditions, product-ion transitions, and collision energies for both analytes. Multiple Reaction Monitoring (MRM) transitions were selected to maximise the analytical sensitivity whilst simultaneously maintaining a high specificity.(4) Chromatographic separations and signal intensity were continuously assessed throughout the optimisation to ensure reliable quantification of MIT and DIT. Chromatographic analysis was performed on a Luna Omega Polar C18 column (1.6µm, 50 x 2.1 mm). Samples were analysed using a flow rate of 0.400 mL/min with an injection volume of 10 µL. The column temperature was maintained at 30°C throughout analysis, and electrospray ionisation was performed in positive ion mode. Optimisation of mobile phase compositions and gradient programmes was completed.

Results

We optimised the chromatographic conditions, extraction procedures and calibration parameters to improve analytical performance. Successful optimisation of LC-MS/MS and SPE enabled us to successfully quantify MIT and DIT. Initially, a starting gradient of 75% water and 25% methanol was tested; however, neither analyte was retained on the column.

Therefore, we increased the water proportion to 98% water and 2% methanol, which improved retention, but gave a broad chromatographic peak. An optimised gradient starting at 90% water (0.1% formic acid) and 10% methanol to 30% methanol over 3.5 minutes resulted in sharp peaks and improved chromatographic separation **(Figure 1 & 3).**

	Time (min)	Flow (mL/min)	% Water (0.1% formic acid)	% Methanol	Curve
1	Initial	0.400	90.0	10.0	Initial
2	1.50	0.400	90.0	10.0	6
3	3.50	0.400	70.0	30.0	6
4	5.50	0.400	5.0	95.0	6
5	5.60	0.400	90.0	10.0	6
6	6.00	0.400	90.0	10.0	11

Figure 1. Optimised liquid chromatography gradient for analysis of Mit and DIT. Mobile phase A contained water containing 0.1% formic acid and mobile B contained methanol. The assay was performed at a constant flow rate of 0.400mL/min with a starting gradient of 90% mobile phase A and 10% mobile phase B before returning to the initial conditions.

Calibration samples were prepared over a concentration range extending from 1 pg/mL to 100 ng/mL to evaluate assay linearity, plotting peak area ratios between analytes and their corresponding internal standard. A 12-point calibration series was extracted alongside the samples with concentrations of 0, 0.001, 0.005, 0.01, 0.05,0.1, 0.25, 0.5, 1,5,10,50,100 ng/mL.

Extraction of MIT and DIT was optimised. Initially, liquid-liquid (LLE) extraction was used with methyl tert-butyl ether (MTBE), but this resulted in no recovery as both analytes remained in the aqueous phase. We then investigated solid-phase extractions (SPE) using a C18 SPE plate, resulting in low recovery of ~35% for both analytes. Extraction using an Evolute Express AX mixed-mode anion exchange sorbent substantially improved recovery to ~90%. The optimised extraction was, condition the cartridge with methanol, then water, before loading the samples. The cartridge was then washed using ammonium hydroxide in acetonitrile; ammonium hydroxide, methanol and formic acid in dichloromethane. MIT and DIT were subsequently eluted using 5% formic acid in methanol, evaporated under nitrogen at 40°C and reconstituted in 150µL of 50:50 methanol-water containing 0.1% formic acid before LC-MS/MS analysis. This optimised method was applied to mouse thyroid, heart, liver (after homogenisation) and serum samples, as well as human serum and urine samples to determine if MIT and DIT were detectable and quantifiable. The chromatograms were evaluated for retention time, peak shape and signal intensity, and the analyte concentrations were calculated using the calibration curves.

Calibration curves demonstrated excellent linearity across the analytical range for both MIT and DIT, with calibration curves with a coefficient of determination (R^2) >0.999 **(Figure 2).** Thus, confirming the method suitability for quantitative analysis of both analytes in biological samples.

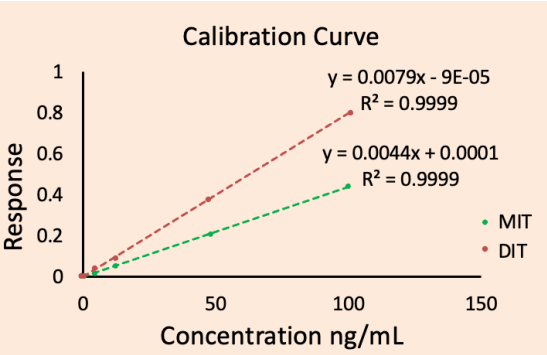


Figure 2. Calibration curves for mono-iodotyrosine (MIT) and diiodotyrosine (DIT) generated using the optimised LC-MS/MS method. Calibration standards ranging from 1 pg/mL to 100 ng/mL. Calibration curves were constructed by plotting analyte response, relative to internal standard against concentration for MIT (green) and DIT (orange). Both analytes demonstrated excellent linearity across the analytical concentration range with coefficients of determination (R2) of >0.999 for both MIT and DIT.

MIT concentrations measured 5.6 ng within thyroid tissue compared with 0.8 ng in heart tissue. DIT concentrations were approximately 19.8 ng in thyroid tissue and 1.7 ng in heart tissue. Chromatograms demonstrated sharp peaks with reproducible retention times for both analytes, confirming that this method successfully quantifies MIT and DIT in tissues **(Figure 3)**.

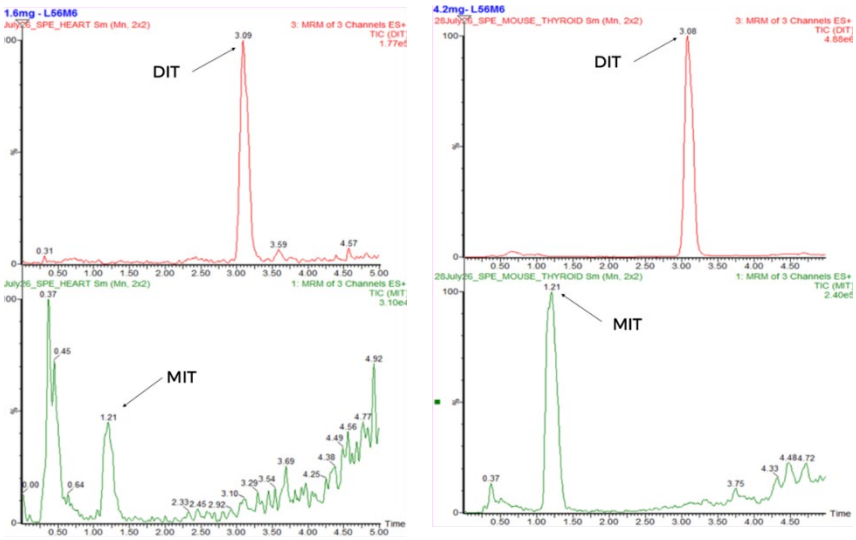


Figure 3. Detection of mono-iodotyrosine (MIT) and di-iodotyrosine (DIT) in mouse thyroid and heart tissue using the optimised LC-MS/MS method.) Chromatograms show detection of DIT (red, upper panels) and MIT (green, lower panels) following solid-phase extraction. **(left)** Mouse heart tissue with MIT (retention time 1.21 mins) and DIT (3.09 mins). **(right)** Mouse thyroid tissue showing MIT and DIT

Discussion

We successfully developed an LC-MS/MS method for the quantification of the thyroid hormone precursors mono-iodotyrosine (MIT) and di-iodotyrosine (DIT). The assay developed during this studentship represents a starting point for the expansion of thyroid hormone metabolic profiling.

One of the major challenges encountered during the project was the efficient extraction of MIT and DIT. The analytes high polarity limited their partitioning into the organic solvent, and so we had to optimise a solid-phase extraction method. The optimisation of solid-phase extraction sorbent and wash steps improved recovery from ~30% to 90%.

Conclusion

We have developed an LC-MS/MS method for the measurement of MIT and DIT, expanding thyroid hormone metabolomics beyond established hormone measurements. This approach will enable future investigations into thyroid hormone biosynthesis, metabolism, and iodide homeostasis. These findings establish a strong analytical foundation for future validation studies in biological samples. This may improve future investigations into thyroid disorders such as hypothyroidism and hyperthyroidism and other thyroid hormone research.

In addition to the scientific findings, I have gained extensive practical training in analytical chemistry and laboratory research. Throughout the project, I have developed practical experience in LC-MS/MS method development, optimisation of chromatographic conditions, sample preparation using solid-phase extractions, interpretation of chromatographic data and will be presenting my work as a poster at the BMSS 2026 annual conference. These experiences have strengthened both my technical laboratory skills and my interest in pursuing future research within clinical science.

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