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A Mass Spectrometry-Based Panel of Nine Thyroid Hormone Metabolites in Human Serum

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BACKGROUND: While thyroxine (T4), 3,3',5-triiodothyronine (T3), and 3,3',5'-triiodothyronine (rT3) have routine methods available for evaluating patients with suspected thyroid disease, appropriate methods for the measurement of other thyroid hormone metabolites (THMs) are lacking. The effects of other iodothyronines or iodothyroacetic acids are therefore less explored. To better understand the (patho)physiological role of THMs, a robust method to measure iodothyronines and iodothyroacetic acids in serum in a single analysis is needed, including associated reference intervals.

METHODS: Clinical and Laboratory Standards Institute guidelines, European Medicines Agency guidelines, and the National Institute of Standards and Technology protocol were used for the method validation and reference intervals. Reference intervals were determined in 132 healthy males and 121 healthy females. Serum samples were deproteinized with acetonitrile, followed by anion-exchange solid phase extraction and analysis with LC-MS/MS, using eight $^{13}C_6$-internal standards

RESULTS: The analytical method validation was performed for all nine THMs. Reference intervals (2.5th to 97.5th percentile) were determined for L-thyronine (4.9–11.3 ng/dL), 3-monoiodothyronine (0.06 –0.41 ng/dL), 3,5-diiodothyronine (<0.13 ng/dL), 3,3'-diiodothyronine (0.25–0.77 ng/dL), T3 (66.4–129.9 ng/dL), rT3 (15.0–64.1 ng/dL), T4 (4.3–10.0 µg/dL), triac/3,3',5-triiodothyroacetic acid (not detected), and tetrac/3,3',5,5'-tetraiodothyroacetic acid (2.2–27.2 ng/dL).

CONCLUSIONS: A broad dynamic concentration range exists among the nine THMs. This method should help to develop a better understanding of the clinical relevance of other THMs, as well as an understanding of thyroid hormone metabolism in health and disease.

Introduction

Thyroid hormones (THs) regulate differentiation, growth, and metabolism in almost all tissues *(1)*. THs are regulated by the hypothalamic-pituitary-thyroid axis, in which pituitary thyroid-stimulating hormone (TSH) stimulates the thyroid to produce predominantly the prohormone thyroxine (T4) and to a lesser extent the biologically active hormone 3,3',5-triiodothyronine (T3). The iodothyronines T3 and 3,3',5'-triiodothyronine (rT3) and subsequently 3,3'-diiodothyronine (3,3'-T2), 3,5-diiodothyronine (3,5-T2), 3-iodothyronine (3-T1), and L-thyronine (T0) are produced by deiodination, which is considered the most prominent metabolic pathway (Fig. 1)*(2)*. Most studies in humans focus on the effects of T4, T3, and rT3, leaving the effects of other TH metabolites (THMs) largely unknown, in part due to the lack of reliable analytical methods. In animal models, a potential role for 3,5-T2 and 3,3'-T2 in energy metabolism has been proposed *(3, 4)*. An alternative metabolic pathway results in the formation of the iodothyroacetic acids 3,3',5-triiodothyroacetic acid (TA3) and 3,3',5,5'-tetraiodothyroacetic acid (TA4) via oxidative deamination (Fig. 1)*(2)*. For TA3, thyromimetic effects on TSH, neuronal differentiation, skeletal metabolic activity, and hepatic response have been proposed *(5–7)* and TA4 inhibits TSH, stimulates aerobic glycolysis,

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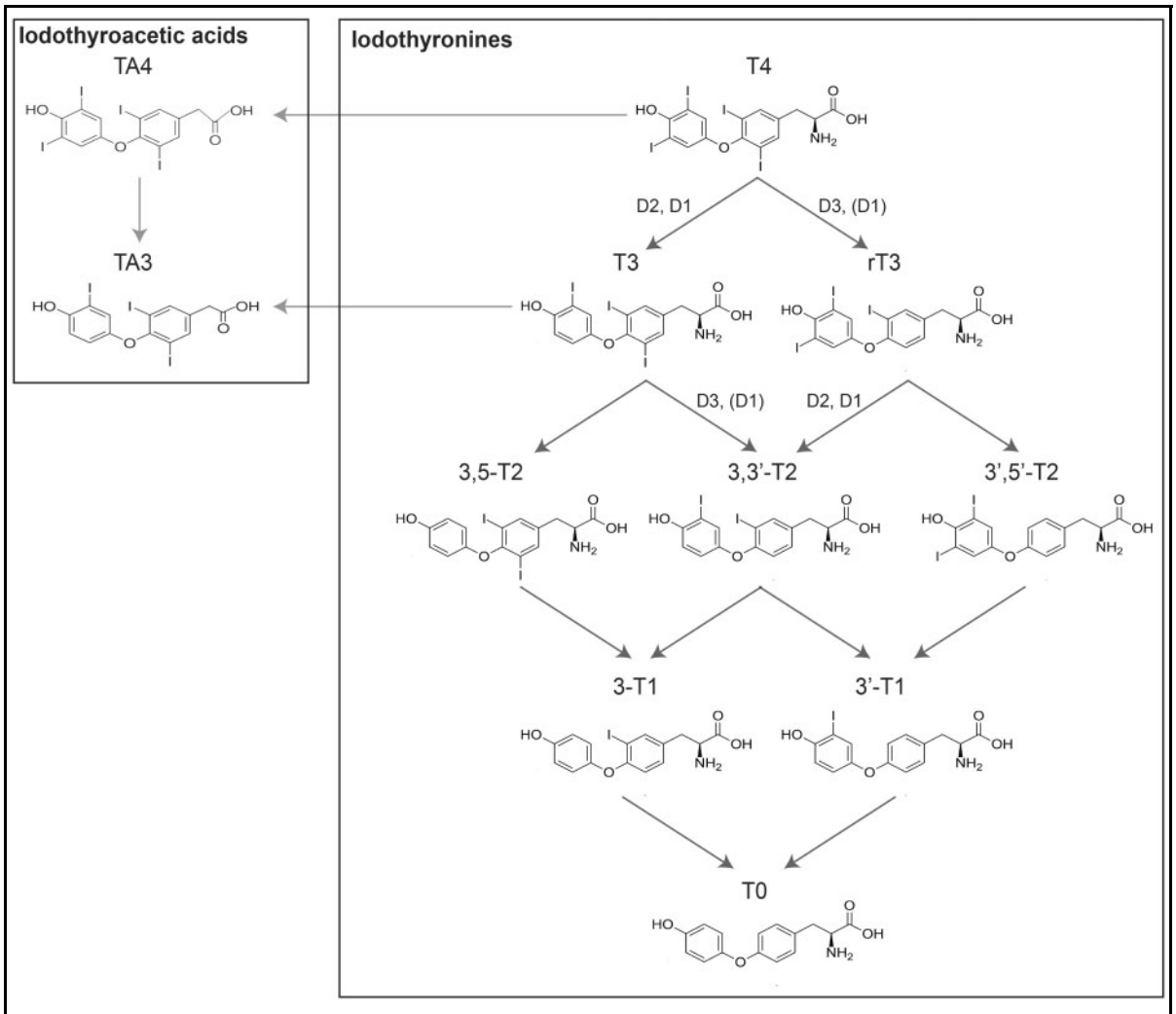

Fig. 1. Schematic overview of thyroid hormone metabolism. The most prominent thyroid hormone metabolic pathway is deiodination by deiodinases, which forms iodothyronine metabolites. Iodothyroacetic acids are formed via oxidative deamination by an alternate metabolic pathway.

induces mild cardiac hypertrophy, and is increased in patients with Graves' disease *(8–11)*.

Several in-house radioimmunoassays have been developed for 3-T1, 3,5-T2, 3,3'-T2, TA3, and TA4 in human serum to study their physiological role (Supplemental Table 1). Interpretation of present studies is impeded due to large variations in observed concentrations resulting from nonselective antibodies and nonstandardized methods. These limitations can be overcome by mass spectrometry (MS). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) can determine several compounds in a single analysis, is very selective, and has less analytical interference compared to radioimmunoassays.

For THMs in human serum, LC-MS/MS methods have mainly focused on single compounds and small iodothyronine panels excluding T0, TA3, and TA4 (Supplemental Table 1)*(11–22)*. Here we developed an LC-MS/MS panel for seven iodothyronines and two iodothyroacetic acids in human serum. Although reference intervals for 3,3'-T2, T3, rT3, and T4 in healthy adults measured with LC-MS/MS have been reported, data on the distribution, exclusion criteria, and gender dependency were not provided nor was the guideline used to establish the reference intervals mentioned (Supplemental Table 1)*(18)*. Therefore, we determined reference intervals in healthy individuals according to Clinical and Laboratory Standards Institute

(CLSI) guidelines for correct interpretation of concentrations in studies.

Materials and Methods

CHEMICALS

Ultra-performance liquid chromatography (UPLC)-MS-grade acetonitrile (ACN), methanol (MeOH), and formic acid (FA) were obtained from Biosolve. A Milli-Q water purification system from Merck-Millipore was used to prepare de-ionized water (>18.2 MΩcm). T4, rT3 and T3 (100 µg/mL in MeOH with 0.1 N NH_3, all purity ≥98.0%), T0 (purity ≥98.0%), ammonia solution 25%, citric acid, ascorbic acid, and dithiothreitol were obtained from Sigma-Aldrich. 3-T1, 3,5-T2, 3,3'-T2, TA3, and TA4 were obtained from Toronto Research Chemicals (all purity ≥95%). 3,3'-T2-$^{13}C_6$, T3-$^{13}C_6$, rT3-$^{13}C_6$, and T4-$^{13}C_6$ (all purity ≥95%) were obtained from Isosciences, and T0-$^{13}C_6$, 3,5-T2-$^{13}C_6$, TA3-$^{13}C_6$, and TA4-$^{13}C_6$ (all purity ≥95%) were obtained from Mercachem.

CALIBRATORS, QUALITY CONTROLS, INTERNAL STANDARD (IS) SOLUTIONS AND ANTI-OXIDANT MIXTURE

Stock solutions (100 µg/mL) of the compounds and IS were prepared in MeOH with 0.1 M NH_3 for iodothyronines and in MeOH for iodothyroacetic acids and stored in amber glass vials (Waters) at -80°C. To prepare eight stock calibrators, two working solutions in MeOH were prepared with different concentrations of nine THMs and serially diluted three times in MeOH (1:1.5 (v/v)) (Supplemental Table 2) before storage in glass vials (Waters) at -20°C. During every sample preparation, calibrators were diluted (1:19 (v/v)) in 50% MeOH. Quality control (QC) samples (600 µL aliquots) were prepared from serum pools spiked with THMs, transferred to 1.5 mL cryotubes and stored at −80°C. To minimize conversion of THMs, an antioxidant mixture was used that consisted of citric acid, ascorbic acid, and dithiothreitol in MilliQ (25 mg/mL each) as previously described (Supplemental Table 2) *(14)*. An IS-mixture was freshly prepared for every sample preparation (Supplemental Table 2). T0-$^{13}C_6$ was used as IS for 3-T1, since a stable isotope-labeled 3-T1-IS was not commercially available.

SAMPLE PREPARATION

Serum, calibrator, or QC (500 µL) was mixed with 50 µL IS mixture and 120 µL antioxidant mixture in a glass tube (75 x 12.00 x 0.8-1.0 mm) and left to equilibrate for 1 hat 37°C. Subsequently, samples were deproteinized with 1.9 mL ACN and stored for 30 min at -20°C before centrifugation (10 min, 3,846 *g*, 10°C). The supernatant was transferred to a new glass tube (75 x 12.00 x 0.8-1.0 mm) and evaporated to dryness under a stream of nitrogen at 50°C. The sample was reconstituted in 450 µL 5% MeOH with 2% ammonia solution in MilliQ (v/v/v). Solid phase extraction 96-well plate cartridges (Waters Oasis MAX, 30 mg, 30 µm) were conditioned (1 mL MeOH) and equilibrated (1 mL MilliQ) before applying the sample. The cartridges were washed (twice 1 mL MilliQ and twice 1 mL MeOH) and THMs were eluted from the cartridge with 500 µL 5% FA in MeOH (v/v) into a 96-well plate with single use glass inserts. The eluate was evaporated to dryness under a stream of nitrogen at 50°C. After reconstitution in 55 µL of 10% ACN with 0.1% ammonia solution, samples were centrifuged (5 min, 939 *g*, 10°C).

LC-MS/MS PROCEDURE

After sample preparation, 35 µL was injected using a Shimadzu Nexera X2 SIL-30AC autosampler at a flow rate of 0.075 mL/min onto a Waters Acquity UPLC BEH C18 column (130 Å, 1.7 µm, 1.0x100 mm) maintained at 40°C using a Shimadzu CTO-20AC column oven. To obtain baseline separation, a gradient of mobile phase A (0.1% FA in MilliQ (v/v)) and mobile phase B (0.1% FA in ACN (v/v)) was used and delivered by a Shimadzu Nexera X2 LC-30AD UPLC system (Supplemental Table 3). The total analysis time was 39 min, including column re-equilibration. A Sciex QTRAP 6500+ was used to measure T0, 3-T1, 3,5-T2, 3,3'-T2, T3, rT3, and T4 with electrospray ionization (ESI) in positive mode and TA3 and TA4 with ESI in negative mode. Mass spectrometer parameters were optimized for each THM by infusion of the respective standard (Supplemental Table 4). Analyst 1.7 software and multiQuant 3.0.2 software packages were used for data analysis. Peak area ratios of compound quantifier and IS and linear regression (line weighting 1/x, intercept not forced through zero) were used to construct calibration curves.

METHOD VALIDATION

Method validation was performed following the CLSI guidelines: EP06-A *(23)* for linearity, CLSI C62A *(24)* for the intra-assay precision, recovery and interferences, and CLSI EP28-A3c *(25)* for reference intervals. The European Medicines Agency guideline *(26)* was followed for carry-over, the inter-assay precision, the lower limit of quantification (LLoQ), matrix effect, and stability and the National Institute of Standards and Technology (NIST) protocol for accuracy *(27)*.

LLOQ AND LINEARITY

To determine the LLoQ, the following concentration ranges were measured (five concentrations, ten times)

for T0 (0.3–5.6 ng/dL), 3-T1 (0.00.8 ng/dL), 3,5-T2 (65–1100 pg/dL), 3,3'-T2 (49–790 pg/dL), T3 (2.9–46.7 ng/dL), rT3 (2.0–33.3 ng/dL), T4 (0.08–1.3 μg/dL), TA3 (1.6–25 ng/dL), and TA4 (8.2–130.8 ng/dL). The LLoQ was defined as the concentration with a minimum signal-to-noise ratio of 10:1, an imprecision (%CV) <20% and a bias <20%. Linearity (nine concentrations, four times) was determined in 50% MeOH and defined as a correlation coefficient (r^2) above 0.99 and a lack-of-fit (*F*-value) below 2.36.

PRECISION AND ACCURACY

The intra-assay precision (five concentrations, twenty times in a single analysis) and the inter-assay precision (three concentrations, in duplicate on 6 days) were determined in serum. Intra-assay precision serum pools were not spiked or spiked with a very low (LL), low (L), medium (M), and high (H) concentration of all nine THMs and inter-assay precision serum pools were spiked with a low (L), medium (M) and high (H) concentration. To determine accuracy, T3 and T4 certified reference material (NIST SRM 971) was measured nine times and bias was statistically calculated according to the NIST protocol *(27)*. Certified reference materials for the other THMs were not available. Accuracy of the other THMs was assessed by comparing the spiked THM concentration with the measured THM concentration corrected for the endogenous THM concentration.

MATRIX EFFECT AND RECOVERY

To determine matrix effect, a spike-order experiment was performed with 50% MeOH and serum from 10 males and 10 females at four concentrations. Compound and IS were spiked after sample preparation. The slope of the calibration curve for each THM in different serum samples was compared to the slope in 50% MeOH. A matrix effect +/- 15% was accepted with a CV below 15%. The recovery was determined in male and female serum pools (three concentrations, in triplicate). Serum pools were divided into aliquot A (spiked with compound and IS before sample preparation) and B (spiked with compound before sample preparation and with IS after sample preparation). For the recovery, the compound/IS ratio of A and B were divided and multiplied with 100% and a CV below 15% was accepted.

INTERFERENCES AND CARRY-OVER

To monitor interference from unknown compounds, the quantitative ion (QN) and the qualitative ion (QL) ratio and limits were determined with the linearity experiment and used to identify potential interferences from unknown compounds in samples (Supplemental Table 5). To monitor interference from known isobaric compounds, retention times of 3'-monoiodothyronine (3'-T1) (60 ng/dL) and 3',5'-diiodothyronine (3',5'-T2) (52.6 ng/dL) were determined. Two transitions from lysophospholipids (496.0 > 184.0, 524.0 > 184.0) and two transitions from phospholipids (758.0 > 184.0, 786.0 > 184.0) were added to the method to determine possible ion suppression regions and potential interference with THMs. To determine carry-over, ten spiked serum samples with 44 ng/dL T0, 6 ng/dL 3-T1, 21.1 ng/dL 3,5-T2, 13.2 ng/dL 3,3'-T2, 400 ng/dL T3, 166.7 ng/dL rT3, 27.2 μg/dL T4, 312.5 ng/dL TA3 and 384.6 ng/dL TA4 were measured followed by the measurement of three blanks. The area of the first blank was compared to the area of the lowest calibrator and a carry-over below 20% for the compound and below 5% for the IS was accepted.

STABILITY

For stability, two serum pools were spiked with a low (L) and high (H) THM concentration. Stability of extracted and nonextracted serum samples (L and H in triplicate) was determined. The storage conditions for extracted samples were 72 h in the autosampler (reconstituted, 10°C) or 72 h in a refrigerator (not reconstituted, 8°C), and for nonextracted samples stability was determined after one, two and three freeze-thaw cycle(s). All samples were compared to fresh samples and considered stable if the difference of the mean concentration was below 15%.

REFERENCE INTERVALS

Between May 2018 and July 2018, whole-blood donor samples [plain tubes (Becton, Dickinson and Company Vacutainer)] were obtained from 253 healthy individuals (132 male, 121 female) at the Blood bank (Sanquin Blood Supply Foundation, Amsterdam, The Netherlands). At Sanquin, donors with autoimmune diseases, epilepsy, heart and vascular disease, and malignant diseases were excluded. Additional exclusion criteria for the inclusion were a history of thyroid disease and use of medication. Samples were fresh residual specimens from daily routine analyses, which were adequately anonymized and blinded after taking note of age and gender. Samples were handled in agreement with the Federation of Dutch Medical Scientific Societies Code of Conduct for responsible use. Donors had the option to declare a no cooperation statement for this procedure. Written informed consent was obtained from all participants. All samples were collected between 9:00 AM and 12:30 PM and centrifuged within 30 min (10 min, 3,846 *g*, 10°C). Serum aliquots (600 μL) were stored at -80°C until analysis.

STATISTICAL ANALYSIS

Data analysis was performed with Analyse-it Software (version 2.30) for Excel and R software with R-package "referenceIntervals" (version 3.5.2) *(28)*. Histogram and

quantile-quantile (Q-Q) plots were used to visually examine the Gaussian probability distribution of the THMs. The CLSI-EP28-A3c guideline *(25)* and nonparametric statistics were used to determine the reference interval. Outliers were excluded with the Horn using Tukey method. Further data analysis was performed on the data set without outliers. Quantile regression (2.5^{th} percentile and 97.5^{th} percentile) was used to determine gender and age dependency. The difference was statistically significant if zero was outside the 90% confidence interval (CI) of the regression slope. To determine correlations between THMs, the Spearman rank-order correlation test was used with a significant correlation at *P*-values below 0.05.

Results

Chromatographic baseline separation of nine THMs was achieved in 22.0 min and enabled the separation of the isobaric compounds T3 and rT3 as well as 3,5-T2 and 3,3'-T2, making accurate quantification possible (Fig. 2 and Supplemental Fig. 1).

METHOD VALIDATION

LLoQ and linearity

The LLoQ was determined for nine THMs (Table 1). A broad concentration range was observed between the nine THMs. THMs were linear in a broad concentration range as is confirmed with a correlation coefficient (r^2) value above 0.99 for all THMs (Table 1).

Precision and accuracy

Intra-assay imprecision was below 15% for all THMs at five concentrations, except for rT3 at 23.4 ng/dL (15.3%) (Supplemental Table 6). Inter-day imprecision was below 15% for all THMs at three concentrations, except for 3-T1 and rT3 (Supplemental Table 7). 3-T1 inter-day imprecision was below 18% and rT3 below

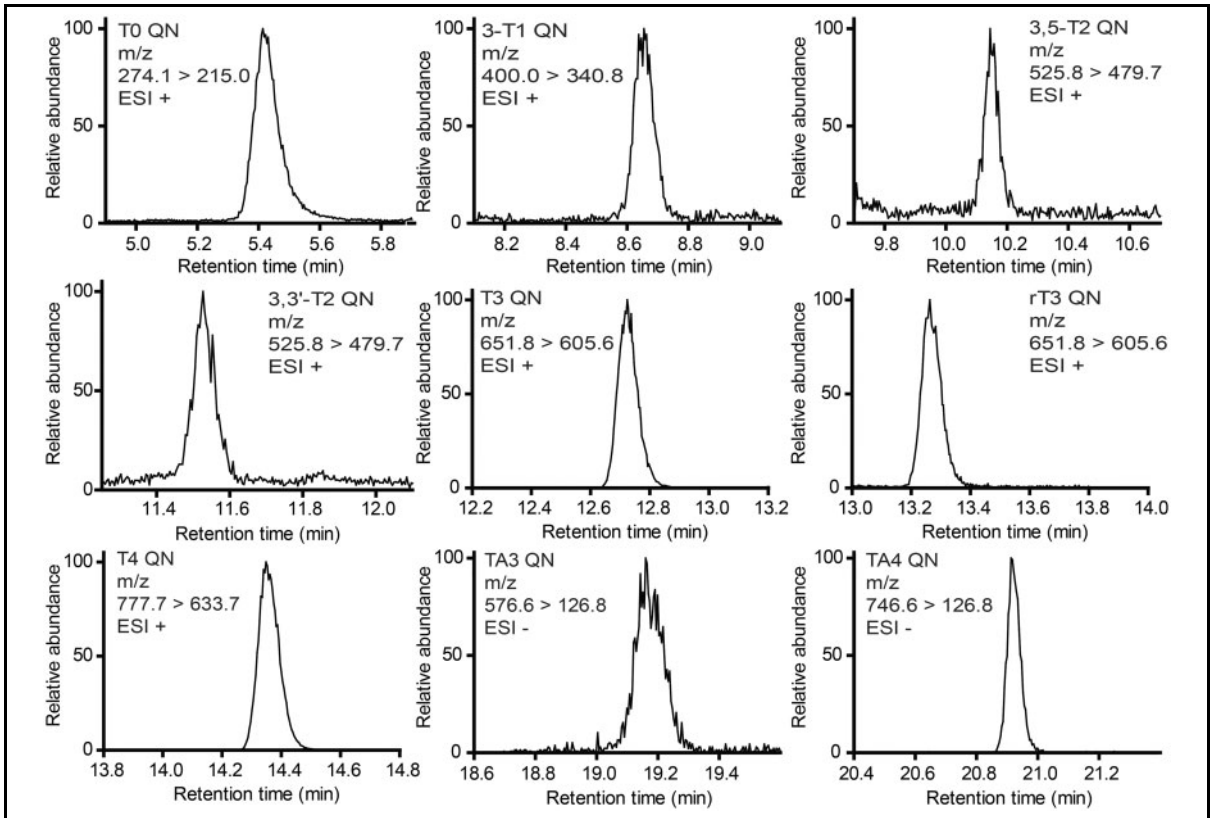

Fig. 2. LC-MS/MS chromatogram of thyroid hormones in spiked serum. Relative abundance of nine thyroid hormone metabolites in serum (containing endogenous concentrations of thyroid hormone metabolites) spiked with 5.6 ng/dL T0, 0.4 ng/dL 3-T1, 0.53 ng/dL 3,3'-T2, 13.3 ng/dL T3, 6.7 ng/dL rT3, 0.8 μg/dL T4, 15.6 ng/dL TA3 and 11.5 ng/dL TA4. QN=quantifier ion, m/z=mass-to-charge, ESI=electrospray ionization, T0=L-thyronine, 3-T1=3-monoiodothyronine, 3,3'-T2=3,3'-diiodothyronine, T3=3,3',5-triiodothyronine, rT3=3,3',5'-triiodothyronine, T4=thyroxine, TA3=triac/3,3',5-triiodothyroacetic acid, TA4=tetrac/3,3',5,5'-tetraiodothyroacetic acid.

Table 1. Method validation parameters.

	Linearity range	Linear regression[a] Y = ax+b (r^2)	Recovery Mean (%CV)	LLoQ	Matrix effect Mean (%CV)
T0	2.7–291.7 ng/dL	Y = 0.2632x + 0.1046 (0.99)	69.6 (6.1)	0.3 ng/dL	101.7 (3.9)
3-T1	0.1–8 ng/dL	Y = 0.0869x - 0.01025 (0.99)	64.6 (13.9)	0.8 ng/dL	54.4 (18.8)
3,5-T2	0.42–21.1 ng/dL	Y = 0.4519x + 0.04356 (0.99)	59.5 (7.1)	65 pg/dL	95.3 (4.0)
3,3′-T2	0.26–23.2 ng/dL	Y = 0.4073x + 0.1263 (0.99)	59.6 (7.0)	49 pg/dL	96.7 (3.8)
T3	16.7–800 ng/dL	Y = 0.03846x + 0.02293 (0.99)	57.1 (7.8)	2.9 ng/dL	110.6 (8.3)
rT3	13.3–1000 ng/dL	Y = 0.03789x - 0.05762 (0.99)	54.2 (15.8)	2.0 ng/dL	103.9 (6.7)
T4	0.26–24.6 µg/dL	Y = 1.536x - 0.05267 (0.99)	49.0 (7.8)	0.32 µg/dL	113.3 (13.2)
TA3	25–650 ng/dL	Y = 0.06491x - 1.113 (0.99)	41.2 (10.9)	12.5 ng/dL	95.9 (4.9)
TA4	22.3–553.8 ng/dL	Y = 0.006194x - 0.01087 (0.99)	38.6 (13.2)	8.0 ng/dL	95.6 (5.7)

[a]y, measured concentration; x, compound area/IS area. *r*, correlation coefficient, CV, coefficient of variation. LLoQ, lower limit of quantification. Conversion factor from ng/dL to pM for T0, 3-T1, 3,5-T2, 3,3′-T2, TA3 and TA4 is 36, 25,19, 19, 16, and 13, respectively; from ng/dL to nM for T3 and rT3 is 0.015 and 0.015, respectively, and from µg/dL to nM for T4 is 12.87. T0 = L-thyronine, 3-T1 = 3-monoiodothyronine, 3,5-T2 = 3,5-diiodothyronine, 3,3′-T2 = 3,3′-diiodothyronine, T3 = 3,3′,5-triiodothyronine, rT3 = 3,3′,5′-triiodothyronine, T4 = thyroxine, TA3 = triac/3,3′,5-triiodothyroacetic acid, TA4 = tetrac/3,3′,5,5′-tetraiodothyroacetic acid.

19%. While no significant bias was observed for T3 in male and female serum, a bias of 0.6 µg/dL was observed for T4.

Matrix effect and recovery

Matrix effect was <15% for all THMs, except for 3-T1, which showed a matrix effect of 45% (Table 1). The recoveries were >38% with CVs <15% for all THMs, except for rT3 (CV 15.8%) (Table 1).

Interferences and carry-over

Ion suppression regions of various (lyso)phospholipids were determined and did not interfere with the measurement of any of the nine THMs. Also 3'-T1 and 3',5'-T2 did not interfere with nine THMs (Supplemental Fig. 2). Carry-over of the compound was below 20% and below 5% for the internal standard for all THMs (Supplemental Table 8).

Stability

Extracted samples stored for 72 h in the autosampler (reconstituted) or in a refrigerator (not reconstituted) were stable compared to fresh samples (Supplemental Fig. 3). Three freeze-thaw cycles did not have an effect on any THM except TA4 (Supplemental Fig. 3). For TA4, we found that more than two freeze-thaw cycles should be avoided due to differences above 15% compared to fresh samples.

Reference intervals with gender and age dependency

We included 253 healthy individuals (132 males, 121 females). The mean age was 51 y (range 20 to 71 y) for males and 39 y (range 19 to 71 y) for females. Non-Gaussian distributions were evident from histograms and Q-Q plots (Supplemental Figs. 4 and 5). Reference intervals for T0, 3-T1, 3,3'-T2, T3, rT3, T4, and TA4 were therefore determined with nonparametric statistics (Table 2). In our reference group, 3,5-T2 concentrations for some samples ranged from 0.02 ng/dL to 0.09 ng/dL (signal-to-noise ratio from 3.4 to 11.9) but with most samples 3,5-T2 was not detected. TA3 was not detected with this method and could therefore not be quantified in the healthy adult group. No subjects had high concentrations for all the THMs measured.

Separate reference intervals were determined for males and females (Table 2). At the 2.5th percentile, no significant differences (90% CI=-0.65 to 0.68 for T0, 90%CI=-0.0094 to 0.0125 for 3-T1, 90% CI=-0.022 to 0.027 for 3,3'-T2, 90% CI=-1.7 to 13.4 for T3, 90% CI=-0.76 to 0.13 for T4, and 90% CI=-0.047 to 0.587 for TA4) between males and females were observed for all THMs, except for rT3 (2.77 ng/dL lower in males, 90% CI=-4.38 to -1.15) (Fig. 3). At the 97.5th percentile, significantly lower concentrations (90% CI=-3.63 to -0.48 for T0, 90% CI=-0.15 to -0.05 for 3-T1, 90% CI=-0.49 to -0.25 for 3,3'-T2, 90% CI=-41.2 to -27.5 for T3, 90% CI=-38.06 to -12.95 for rT3, and 90% CI=-2.4 to -1.4 for T4) were observed in males compared to females for all THMs except for TA4, which was higher in males (90% CI = 13.91 to 77.98) (Fig. 3).

For age at the 2.5th percentile, a significant increase was observed for T0 (90% CI = 0.0087 to 0.0478), 3-T1 (90% CI = 0.00001 to 0.00073), and TA4 (90% CI = 0.0055 to 0.0206) and a significant decrease was observed for T3 (90% CI=-0.37 to -0.16) with increasing age (Supplemental Fig. 6). At the 97.5th percentile, a significant increase was observed for TA4 (90% CI = 0.34

Table 2. Nonparametric gender-dependent reference intervals of nine thyroid hormone metabolites ($n = 253$).

Compound	*n*	Mean (SD)	2.5th percentile (90% CI)	25th percentile	Median	75th percentile	97.5th percentile (90% CI)
T0 (ng/dL)							
Overall	250	7.9 (1.7)	5.0 (4.7–5.3)	6.6	7.7	9.0	11.5 (10.7–12.5)
Male	131	7.6 (1.4)	5.0 (4.7–5.2)	6.4	7.5	8.5	10.6 (10.2–11.6)
Female	121	8.2 (2.0)	4.8 (3.6–5.6)	6.7	8.0	9.6	13.3 (11.4–14.3)
3-T1 (ng/dL)							
Overall	252	0.188 (0.10)	0.059 (0.050–0.064)	0.096	0.182	0.256	0.407 (0.366–0.483)
Male	131	0.197 (0.09)	0.059 (0.042–0.069)	0.120	0.203	0.257	0.365 (0.336–0.372)
Female	121	0.177 (0.11)	0.058 (0.036–0.063)	0.088	0.133	0.244	0.482 (0.397–0.502)
3,5-T2 (pg/dL)#	NA	< 65	< 65	< 65	< 65	< 65	< 65
3,3′-T2 (ng/dL)							
Overall	248	0.43 (0.13)	0.25 (0.23–0.26)	0.34	0.40	0.49	0.77 (0.69–0.91)
Male	132	0.40 (0.10)	0.24 (0.22–0.25)	0.34	0.39	0.46	0.60 (0.58–0.65)
Female	119	0.46 (0.17)	0.24 (0.21–0.28)	0.34	0.43	0.53	0.91 (0.67–1.04)
T3 (ng/dL)							
Overall	246	92.9 (14.9)	66.4 (63.7–71.0)	82.1	91.6	101.6	129.9 (126.0–134.2)
Male	131	90.1 (11.2)	66.3 (63.7–73.2)	81.9	90.3	97.7	112.1 (108.1–122.7)
Female	120	97.0 (20.3)	63.6 (59.6–69.1)	82.4	94.1	106.4	147.5 (131.7–152.2)
rT3 (ng/dL)							
Overall	252	31.0 (13.0)	15.0 (13.9–16.7)	22.3	27.8	36.7	64.1 (57.3–78.0)
Male	132	27.7 (9.4)	13.4 (12.1–15.6)	21.0	25.8	31.6	53.2 (49.0–57.3)
Female	121	34.5 (15.4)	16.2 (14.4–17.6)	22.8	31.3	42.7	77.8 (63.4–92.4)
T4 (μg/dL)							
Overall	253	6.6 (1.4)	4.3 (4.2–4.7)	5.5	6.3	7.4	10.0 (9.2–10.4)
Male	132	6.2 (1.1)	4.2 (3.9–4.6)	5.3	6.1	7.0	8.6 (8.3–9.4)
Female	121	7.0 (1.6)	4.6 (4.2–4.9)	5.7	6.8	8.0	10.4 (9.8–12.5)
TA3	NA	ND	ND	ND	ND	ND	ND
TA4 (ng/dL)							
Overall	245	7.0 (6.6)	2.3 (2.1–2.5)	3.8	4.9	7.0	27.8 (21.7–44.8)
Male	131	9.4 (14.3)	2.4 (2.1–2.7)	3.9	5.3	8.0	54.4 (30.1–104.7)
Female	115	5.9 (3.8)	2.2 (1.9–2.5)	3.8	4.8	6.7	20.8 (19.1–27.9)

NA, not applicable; # below LLoQ, ND, not detected, *n*, Total. Conversion factor from ng/dL to pM for T0, 3-T1, 3,5-T2, 3,3′-T2 and TA4 is 36, 25,19, 19, and 13, respectively; from ng/dL to nM for T3 and rT3 is 0.015 for both, and for T4 from μg/dL to nM is 12.87. T0 = L-thyronine, 3-T1 = 3-monoiodothyronine, 3,5-T2 = 3,5-diiodothyronine, 3,3′-T2 = 3,3′-diiodothyronine, T3 = 3,3′,5-triiodothyronine, rT3 = 3,3′,5′-triiodothyronine, T4 = thyroxine, TA3 = triac/3,3′,5-triiodothyroacetic acid, TA4 = tetrac/3,3′,5,5′-tetraiodothyroacetic acid.

to 2.05) and a significant decrease was observed for T3 (90% CI=-0.77 to -0.43) and T4 (90% CI=-0.07 to −0.01) with increasing age (Supplemental Fig. 6).

Correlation between THMs

Significant correlations were observed between the following THMs and their precursor: T3, rT3, and TA4 with T4 ($r = 0.64$, 0.61, and 0.21, respectively, $P = <2.2*10^{-16}$ for T3 and rT3 and $P = 0.00094$ for TA4); 3,3'-T2 with T3 and rT3 ($r = 0.4$ and 0.67, $P = 5.8*10^{-11}$ and $P = < 2.2*10^{-16}$, respectively); T0 with 3-T1 ($r = 0.33$, $P = 6.2*10^{-8}$) (Fig. 4 and Supplemental Figs. 7 and 8).

Discussion

We developed an LC-MS/MS method that enables the measurement of seven iodothyronines and two

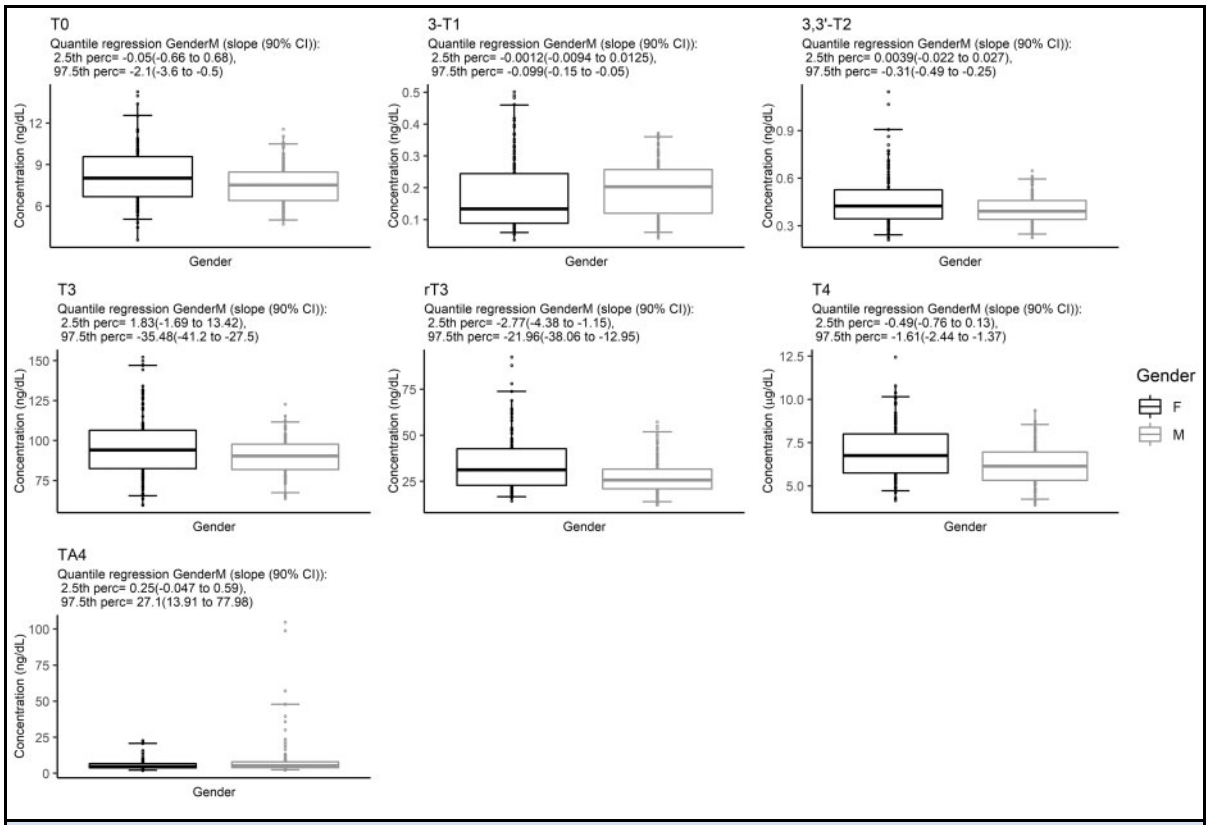

Fig. 3. Thyroid hormone metabolites are dependent on gender. Boxplots (25th, median and 75th percentile) of seven thyroid hormone metabolites with horizontal lines representing the 2.5th percentile and 97.5th percentile. No significant differences between males and females at the 2.5th percentile, except for rT3. Significant lower levels at the 97.5th percentile in males compared to females for all thyroid hormone metabolites except for TA4, which was higher in males. M=male, F=female, rT3=3,3′,5′-triiodothyronine, TA4=tetrac/3,3′,5,5′-tetraiodothyroacetic acid.

iodothyroacetic acids with diverse chemical properties in a single analysis in human serum, which was validated according to CLSI and European Medicines Agency guidelines. In this method extensive sample preparation and the use of $^{13}C_6$-labelled internal standards for eight out of nine THMs was very important.

Our method is unique in that it contains three additional THMs compared to previously published THM panels, including two iodothyroacetic acids *(15, 17–22)*. This panel is essential to better understand the role of endogenous THMs besides T4, T3, and rT3 in healthy and diseased conditions. The added value of this panel in clinical practice and specific patient groups can be determined with clinical validation.

Reference intervals for all nine THMs were established in healthy individuals according to CLSI guidelines. A broad dynamic concentration range between the nine THMs was observed from 0.06 ng/dL to 10.0 µg/dL. T3, rT3, and T4 from our reference group are consistent with reported reference intervals *(18)*. Large variations in concentrations have been published for 3,5-T2 and 3,3'-T2 due to nonselective antibodies and/or nonstandardized methods (Supplemental Table 1) *(29–31)*. The mass spectrometer cannot distinguish between the isobaric compounds 3,5-T2, 3,3'-T2, and 3',5'-T2, and therefore adequate and reproducible baseline separation by liquid chromatography is essential for accurate quantification. Our LC-MS/MS method separates 3',5'-T2 at baseline from 3,5-T2 and 3,3'-T2, as confirmed by our interference experiment. In the reference group, 3,5-T2 concentrations ranged from no detection to 0.09 ng/dL and 3,3'-T2 concentrations ranged from 0.25 to 0.77 ng/dL. Remarkably, both reference intervals are substantially lower compared to previously published concentrations *(18, 20, 21)*. Higher concentrations of 3,5-T2 and 3,3'-T2 might be ascribed to less optimized liquid chromatography, contamination of 3,5-T2 in the $^{13}C_9$-^{15}N-labelled 3,5-T2 internal standard and/or the selected (reference) population *(18, 20, 21)*. In our reference group we could not detect

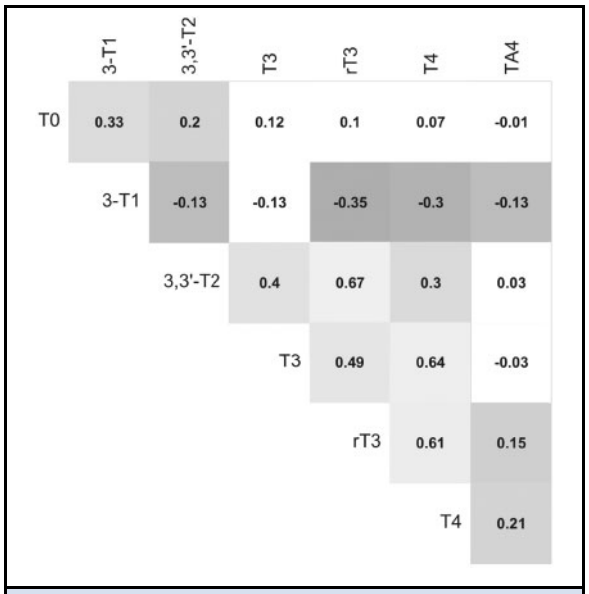

Fig. 4. Correlations between thyroid hormone metabolites and their precursor. Correlation matrix of the thyroid hormone metabolites measured with LC-MS/MS. Spearman rank-order correlation's coefficient is shown in the matrix with a positive correlation in light gray and a negative correlation in dark gray. The Spearman rank-order correlation test was used to determine if the correlation was significant (*P*-value <0.05). All significant correlations have a shaded gray background.

concentrations TA3 above our LLoQ of 12.5 ng/dL, while concentrations up to 15.2 ng/dL have been measured by other groups *(32–34)*. However, our developed method enables us to monitor patients with thyroid hormone transporter monocarboxylate transporter 8 deficiency on TA3 treatment *(35)* and provides an opportunity to study THM metabolism in TA3 treated patients in future studies. In our large reference group of 253 healthy individuals, TA4 ranged from 2.3 to 27.8 ng/dL, in agreement with the variation observed in euthyroid patient cohorts in literature *(11, 36–39)* (Supplemental Table 1). It has been suggested that the binding protein transthyretin may cause this variation, but TA4 was not significantly correlated ($r < 0.03$) with transthyretin in serum in a previous study *(38)*.

Separate reference intervals for males and females are provided in this study since gender dependency was observed for all THMs. Thyroid binding proteins might cause this gender dependency. Age dependency has been reported for 3,3'-T2, T3, and T4 and concentrations decreased with increasing age *(18)*. In our reference group, we did not observe age dependency for 3,3'-T2, which might be ascribed to our sample size.

THMs are formed from specific precursors via metabolic pathways (Fig. 1). We observed that all iodothyronines but one were significantly and positively correlated with their precursor, supporting that this correlation can be used to probe metabolic pathways of THs. Interestingly, 3,3'-T2 and 3-T1 were significantly and negatively correlated, suggesting 3-T1 might have another major precursor in serum. Also, a positive, significant correlation was observed for TA4 with T4, suggesting T4 as a precursor of TA4 in serum as previously reported *(11)*.

A limitation of this LC-MS/MS method is, first, the lack of an internal standard for 3-T1 and the matrix effect of 45% with a CV of 19% using T0-$^{13}C_6$ that could not be overcome. The method for 3-T1 has, however, proved to be selective and sensitive, and has an imprecision of below 8% intra-assay and below 22% inter-assay. Second, the low recovery of TA3 and TA4 can have consequences for the analytical sensitivity and the precision of the method. However, we used $^{13}C_6$-labelled internal standards for TA3 and TA4 correcting for the losses during sample preparation and during sample measurement. Third, although we believe that our reference population was representative of a general nondisease population, we lacked information about usage of over-the-counter medication, supplements, and lifestyle factors that could have impacted the concentrations measured

In summary, we developed and analytically validated an LC-MS/MS method for a panel of nine THMs in human serum. Reliable gender dependent reference intervals were established for T0, 3-T1, 3,3'-T2, T3, rT3, T4, and TA4. Among the THMs a broad dynamic concentration range from 0.06 ng/dL to 10.0 µg/dL was observed. This LC-MS/MS method could add to a better understanding of the role of the different components of the thyroid metabolome in health and disease and may help to clinically identify thyroid diseases in specific patient categories.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard abbreviations: TH, thyroid hormone; TSH, thyroid-stimulating hormone, T4, thyroxine; T3, 3,3',5-triiodothyronine; rT3, 3,3',5'-triiodothyronine; 3,3'-T2, 3,3'-diiodothyronine; 3,5-T2, 3,5-diiodothyronine; 3-T1, 3-monoiodothyronine; T0, L-thyronine; TA3, triac/3,3',5-triiodothyroacetic acid; TA4, tetrac/3,3',5,5'-tetraiodothyroacetic acid; THMs, thyroid hormone metabolites; ACN, acetonitrile; MeOH, methanol; FA, formic acid; IS, internal standard; ESI, electrospray ionization; LLoQ, lower limit of quantification; r^2, Ccorrelation coefficient; QN, quantitative ion; QL, qualitative ion; 3'-T1, 3'-monoiodothyronine; 3',5'-T2, 3',5'-diiodothyronine; Q-Q, quantile-quantile; Log P, logarithm of the partition coefficient.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4*

requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

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