Pediatric Reference Intervals for Free Thyroxine and Free Triiodothyronine

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Background: The clinical value of free thyroxine (FT₄) and free triiodothyronine (FT₃) analysis depends on the reference intervals with which they are compared. We determined age- and sex-specific reference intervals for neonates, infants, and children 0–18 years of age for FT₄ and FT₃ using tandem mass spectrometry.

Methods: Reference intervals were calculated for serum FT₄ ($n = 1426$) and FT₃ ($n = 1107$) obtained from healthy children between January 1, 2008, and June 30, 2008, from Children's National Medical Center and Georgetown University Medical Center Bioanalytical Core Laboratory, Washington, DC. Serum samples were analyzed using isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) with deuterium-labeled internal standards.

Results: FT₄ reference intervals were very similar for males and females of all ages and ranged between 1.3 and 2.4 ng/dL for children 1 to 18 years old. FT₄ reference intervals for 1- to 12-month-old infants were 1.3–2.8 ng/dL. These 2.5 to 97.5 percentile intervals were much tighter than reference intervals obtained using immunoassay platforms 0.48–2.78 ng/dL for males and 0.85–2.09 ng/dL for females. Similarly, FT₃ intervals were consistent and similar for males and females and for all ages, ranging between 1.5 pg/mL and approximately 6.0 pg/mL for children 1 month of age to 18 years old.

Conclusions: This is the first study to provide pediatric reference intervals of FT₄ and FT₃ for children from birth to 18 years of age using LC/MS/MS. Analysis using LC/MS/MS provides more specific quantification of thyroid hormones. A comparison of the ultrafiltration tandem mass spectrometric method with equilibrium dialysis showed very good correlation.

Introduction

IN 1990 THE AMERICAN THYROID ASSOCIATION (ATA) recommended the use of the sensitive thyrotropin (TSH) and free thyroxine (FT₄) tests to evaluate thyroid function (1). In the case of hypothyroidism, serum TSH levels are high and FT₄ levels low, while in subclinical hypothyroidism serum TSH levels are high but FT₄ levels are within the normal reference interval. FT₄ testing is critical when pituitary or hypothalamic disease are known or suspected.

FT₄, the unbound form of the thyroid hormone thyroxine (T₄), is representative of thyroid status. Therefore, serum FT₄ levels reflect the health of the thyroid gland and can assist in thyroid disease diagnosis (2). During fetal development, thyroid hormones are important in brain cell migration as well as differentiation of neurons, oligodendrocytes, astrocytes, and microglia (3). Therefore, adequate levels of maternal T₄ are important for appropriate fetal neurodevelopment (4–9). Neonatal screening can help in the timely diagnosis of

newborn thyroid diseases such as congenital hypothyroidism and thus prevent developmental or growth problems (10).

Approximately 20% of triiodothyronine (T₃), the more active thyroid hormone, is synthesized by the thyroid gland, while 80% is formed in the peripheral tissues from the 5'-deiodination of T₄. About 99.9% of T₃ is normally found in the circulation bound to transport proteins. T₃ has a lower affinity for binding to transport proteins and globulins than T₄ (11).

Population-based reference intervals are a tool for interpretation of individual patient laboratory test results. The clinical value of FT₄ results depends crucially on the reference intervals with which they are compared. Accuracy of FT₄ and FT₃ measurements by the direct analogue immunoassay methods has been questioned by many investigators because FT₄ measurements are still vulnerable to method-dependent artifacts, particularly in some specific populations (12–17). For this reason we elected to assess the pediatric reference intervals using a specific and robust ultrafiltration tandem mass

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FIG. 1. Effect of ultrafiltration temperature on free thyroxine (FT₄) concentrations.

spectrometry (MS/MS) procedure developed in our laboratories (15,17).

Materials and Methods

Blood samples were obtained from Children's National Medical Center's (CNMC), Washington, DC, healthy outpatient population from January 2008 to June 2008. All personal identifiers except age and sex were removed and hormone profiles were performed on plasma or serum samples. Institutional Review Board approval was obtained. Serum TSH levels were within the normal reference intervals for all children (testing performed at CNMC; data not presented). All samples were kept at -80°C until analysis.

Chemicals and reagents

3,3',5-Triido-L-thyronine (T₃) and L-thyroxine (T₄) were purchased from Sigma (St. Louis, MO). Deuterium-labeled T₄ (T₄-d₅): T₄-[tyrosine ²H₅] was obtained from IsoSciences (King of Prussia, PA) and served as the internal standard. High performance liquid chromatography (HPLC) grade methanol and ammonium hydroxide were purchased from Fisher Scientific (Fair Lawn, NJ). HPLC grade glacial acetic acid was obtained from JT Baker (Phillipsburg, NJ). Acetonitrile was purchased from Burdick and Jackson (Muskegon, MI). Distilled de-ionized water was prepared from Millipore RiOs and Milli-Q Gradient ultrapure water system (Billerica, MA). Standards for the calibration curve in the range of 0.1–2.5 ng/dL (1.5–38.5 pmol/L) for FT₃ and 0.5–5 ng/dL (6.3–63.0 pmol/L) for FT₄ were prepared by adding both T₃ and T₄ spiking solutions to distilled de-ionized water. In-house quality control solutions at three concentrations (low, medium, high) were prepared in the same way to evaluate within-day and between-day precision as well as the method accuracy. A diluted solution of 2.5 ng/dL (31.5 pmol/L) T₄-d₅ in methanol was used as working internal standard.

Liquid chromatography tandem mass spectrometry analysis

Thyroid hormones were analyzed by methods developed in our laboratories and described in detail previously (15,17).

FIG. 2. Effect of ultrafiltration temperature on free triiodothyronine (FT₃) concentrations.

Briefly, 400 μL of serum or plasma were centrifuged using 30 kDa cut-off ultrafiltration devices (Millipore, Bedford, MA) to yield ultrafiltrates (200 μL) prepared at either 25°C or 37°C . One hundred fifty microliters of ultrafiltrate was treated with 450 μL methanol containing deuterium-labeled internal standards. The solution was vortexed and centrifuged, 500 μL of the supernatant was treated with 400 μL of water, and 650 μL was injected onto the C-18 column (Supelco LC-18-DB [3.3 cm $\times$ 3.0 mm, 3.0 μm ID]). The thyroid hormones were quantified in the negative mode employing multiple reaction mode monitoring on the API-5000 tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with TurbolonSpray source was operated in the negative ion multiple reaction monitoring mode to perform the analysis. The HPLC system consisted of three Shimadzu LC-20AD pumps, a Shimadzu SIL-HT_A autosampler, and a Shimadzu DGU-20A₅ degasser (Shimadzu Scientific Instruments, Columbia, MD). The liquid chromatography tandem mass spectrometry (LC/MS/MS) procedure involves an online extraction/cleaning of the injected samples followed by an activation of a built-in Valco switching valve for subsequent sample introduction into the mass spectrometer.

FIG. 3. FT₄ analysis using ultrafiltration vs. equilibrium dialysis 37°C $n=37$, $r=0.806$, $\text{Syx}=0.1339$, $y=0.652x+0.212$.

TABLE 1. REFERENCE INTERVALS FOR FREE THYROXINE NG/DL BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Age	n	Reference intervals ultrafiltration at 37°C		Reference intervals ultrafiltration at 25°C	
		2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile
Females and males					
1 month to <1 year	140	1.3	2.8	0.9	1.9
1 to <3 years	145	1.3	2.4	0.9	1.6
3 to <8 years	129	1.3	2.4	0.9	1.6
Females					
8 to <11 years	109	1.3	2.4	0.9	1.6
11 to <14 years	137	1.3	2.4	0.9	1.6
14 to <16 years	119	1.3	2.4	0.9	1.6
16 to <18 years	120	1.3	2.4	0.9	1.6
Males					
3 to <8 years	119	1.3	2.4	0.9	1.6
8 to <12 years	152	1.3	2.4	0.9	1.6
12 to <15 years	135	1.3	2.4	0.9	1.6
15 to <18 years	121	1.3	2.4	0.9	1.6

Ultrafiltration was performed on all samples at 25°C. For comparison purposes the process was repeated on 28 samples at 37°C. This provided a factor allowing conversion of reference intervals derived at 25°C to those at 37°C. Results and comparisons are provided in Figs. 1 and 2.

Precision was evaluated by analyzing in-house quality control samples at three different concentration levels in duplicates for both FT₃ and FT₄ both within and between-day. Accuracy was evaluated by performing recovery studies. The between-day precision was measured on 20 different days for FT₃ and 30 different days for FT₄.

Statistical analysis

The 2.5 and 97.5 percentiles were determined and reference intervals were established for all analytes on this cohort of healthy outpatient pediatric population. Linear regression analysis was performed by using GraphPad Prism version 3.02 (GraphPad Software, San Diego, CA) (Figs. 1–3).

Results

Reference intervals for FT₄ and FT₃ at both 25°C and 37°C are shown in Tables 1 and 2. Results obtained for 28 samples

comparing ultrafiltration at 25°C vs. 37°C are shown in Figs. 1 and 2. Comparison of the ultrafiltration MS/MS procedure with equilibrium dialysis MS/MS procedure is shown in Fig. 3 (both performed at 37°C).

Discussion

A narrow range of reference intervals was found for FT₄ for both males and females. The values did not change according to sex. In the first year of life, the upper bound for FT₄ is slightly higher than FT₄ levels for children of both sexes older than 1 year of age (Table 1). A narrow range of reference intervals was found for FT₄ for both males and females at both ultrafiltration temperatures 25°C and 37°C.

The lower bounds for FT₃ levels were very consistent among males and females and did not fluctuate with age. The upper bounds of the reference intervals, however, show a slight increase of value in the female group 8 to <13 years and male group 8 to <12 years, as well as males 16 to <18 years (Table 2).

The upper FT₄ value reached a maximum of 1.9 ng/dL when ultrafiltration was performed at 25°C (2.8 ng/dL if performed at 37°C) in the 1 month to <1 year group of both

TABLE 2. REFERENCE INTERVALS FOR FREE TRIIODOTHYRONINE PG/mL BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

Age	n	Reference intervals ultrafiltration at 37°C		Reference intervals ultrafiltration at 25°C	
		2.5 percentile	97.5 percentile	2.5 percentile	97.5 percentile
Females and males					
1 month to <8 years	124	1.5	6.0	1.0	4.0
Females					
8 to <13 years	122	1.5	6.7	1.0	4.5
13 to <15 years	145	1.5	6.3	1.1	4.2
15 to <17 years	187	1.5	6.0	1.1	4.0
17 to <18 years	57	1.5	6.1	1.1	4.1
Males					
8 to <12 years	144	1.5	6.7	1.1	4.5
12 to <14 years	140	1.5	6.2	1.0	4.1
14 to <16 years	102	1.5	6.0	1.1	4.0
16 to <18 years	86	1.5	6.7	1.1	4.5

sexes, and decreased to 1.6 ng/dL (2.4 ng/dL at 37°C) in the 1- to 3-year group of both sexes, and then stabilized to a value of 1.6 ng/dL (2.4 ng/dL at 37°C) for both males and females. The lower values for all ages were slightly higher than the published values conducted by immunoassays (18–20). The initial decrease is consistent with the published literature. However, in the published literature, the upper ranges for free T_4 continue to decrease with age, as opposed to our values, which do not continue to decrease with increasing age, but rather stay consistent after age 3 (18–20). Our results do not indicate a sex difference in the values of serum FT_4 , which is inconsistent with previously published values (18,19).

Free T_3 lower reference ranges were very consistent with no patterns regarding sex or age. This consistency contrasts with the published literature values, which show an increase in the lower bound values with increasing age, and a decrease in the upper bound values after the first month of life (20).

The temperature at which ultrafiltration is performed merits some discussion. When our ultrafiltration MS/MS method was first developed it was compared to the then gold standard Nichols immunoassay kit in which equilibrium dialysis was performed at 37°C. We found a slope of 1 with an excellent correlation with the Nichols approach only if the ultrafiltration was performed at 25°C (17). In a small study, use of 37°C for ultrafiltration increased values of FT_4 by approximately a factor of 1.5 (17). Once the Nichols kit was discontinued large reference laboratories were forced to use a different dialysis membrane and their results at 37°C were also found to be approximately 1.5 times higher than the classical Nichols equilibrium dialysis immunoassay method performed at 37°C. In a larger study reported in this paper we have now found that switching the ultrafiltration from 25°C to 37°C increased our results by a similar factor of 1.5 (Figs. 1 and 2) for both FT_4 and FT_3 .

In this study we have also compared the ultrafiltration MS/MS method with an equilibrium dialysis MS/MS method. Results of this comparison at 37°C are shown in Fig. 3 and show good agreement.

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Disclosure Statement

No competing financial interests exist.

References

1. Surks MI, Chopra IJ, Mariash CN, Nicoloff JT, Solomon DH 1990 American Thyroid Association guidelines for use of laboratory tests in thyroid disorders. *JAMA* **263**:1529–1532.
2. Stockigt JR 2001 Thyroid hormone binding and metabolism. In: Degroot LJ, Jameson JL, editors. *Endocrinology*, 4th ed. W.B. Saunders Co., Philadelphia, pp 1314–1326.
3. Bernal J, Guadano-Ferraz A, Morte B 2003 Perspectives in the study of thyroid hormone action on brain development and function. *Thyroid* **13**:1005–1012.
4. Haddow JE, Palomaki GE, Allan WC, Williams JR, Knight GJ, Gagnon J, O'Heir CE, Mitchell ML, Hermos RJ, Waisbren SE, Faix JD, Klein RZ 1999 Maternal thyroid deficiency during pregnancy and subsequent neuropsychological development of the child. *N Engl J Med* **341**:549–555.
5. Williams GR 2008 Neurodevelopmental and neurophysiological actions of thyroid hormone. *J Neuroendocrinol* **20**: 784–794.
6. Morreale de Escobar G, Calvo R, Obregon MJ, Escobar Del Rey F 1990 Contribution of maternal thyroxine to fetal thyroxine pools in normal rats near term. *Endocrinology* **126**:2765–2767.
7. Mitchell ML, Klein RZ 2004 The sequelae of untreated maternal hypothyroidism. *Eur J Endocrinol* **151**(Suppl 3): U45–48.
8. Obregon MJ, Calvo RM, Del Rey FE, de Escobar GM 2007 Ontogenesis of thyroid function and interactions with maternal function. *Endocr Dev* **10**:86–98.
9. Morreale de Escobar G, Obregon MJ, Escobar del Rey F 2000 Is neuropsychological development related to maternal hypothyroidism or to maternal hypothyroxinemia? *J Clin Endocrinol Metab* **85**:3975–3987.
10. LaFranchi S 1999 Congenital hypothyroidism: etiologies, diagnosis, and management. *Thyroid* **9**:735–740.
11. Wilke TJ 1986 Estimation of free thyroid hormone concentrations in the clinical laboratory. *Clin Chem* **32**:585–592.
12. d'Herbomez M, Forzy G, Gasser F, Massart C, Beaudonnet A, Sapin R 2003 Clinical evaluation of nine free thyroxine assays: persistent problems in particular populations. *Clin Chem Lab Med* **41**:942–947.
13. Ekins R 1987 Validity of analog free thyroxine immunoassays. *Clin Chem* **33**:2137–2144.
14. Ekins R 1993 Analytic measurements of free thyroxine. *Clin Lab Med* **13**:599–630.
15. Gu J, Soldin OP, Soldin SJ 2007 Simultaneous quantification of free triiodothyronine and free thyroxine by isotope dilution tandem mass spectrometry. *Clin Biochem* **40**:1386–1391.
16. Sapin R, d'Herbomez M 2003 Free thyroxine measured by equilibrium dialysis and nine immunoassays in sera with various serum thyroxine-binding capacities. *Clin Chem* **49**: 1531–1535.
17. Soldin SJ, Soukhova N, Janicic N, Jonklaas J, Soldin OP 2005 The measurement of free thyroxine by isotope dilution tandem mass spectrometry. *Clin Chim Acta* **358**:113–118.
18. Ghoshal AK, Soldin SJ 2003 Evaluation of the Dade Behring Dimension RxL: integrated chemistry system-pediatric reference ranges. *Clin Chim Acta* **331**:135–146.
19. Murthy JN, Hicks JM, Soldin SJ 1995 Evaluation of the Technicon Immuno I random access immunoassay analyzer and calculation of pediatric reference ranges for endocrine tests, T-uptake, and ferritin. *Clin Biochem* **28**:181–185.
20. Soldin SJ, Morales A, Albalos F, Lenherr S, Rifai N 1995 Pediatric reference ranges on the Abbott IMx for FSH, LH, prolactin, TSH, T_4 , T_3 , free T_4 , free T_3 , T-uptake, IgE, and ferritin. *Clin Biochem* **28**:603–606.

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