Free Thyroxine Concentrations in Sera of Individuals with Familial Dysalbuminemic Hyperthyroxinemia: A Comparison of Three Methods of Measurement

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Background: Euthyroid individuals with familial dysalbuminemic hyperthyroxinemia (FDH) have often falsely elevated serum free thyroxine (fT4) concentrations determined by different automated immunoassays.

Methods: We measured serum fT4 using direct dialysis coupled with tandem mass spectrometry (fT4 DDMS) in individuals with the common *albumin* gene mutation (*ALB* R218H) from 14 FDH families and compared them with results obtained by direct immunometric assay (fT4 DIMM) and free thyroxine index (fT4I).

Results: While all 14 individuals with FDH had elevated total serum T4, the fT4 measured by DIMM was elevated in 12, by fT4I in 5, and by DDMS in 1.

Conclusion: The latter method greatly reduced the discordance of fT4 results relative to thyrotropin in FDH.

Keywords: FDH, albumin, mutation, mass spectrometry, free T4

Introduction

FAMILIAL DYSALBUMINEMIC HYPERTHYROXINEMIA (FDH) is a dominantly inherited condition caused by gain-of-function mutations in the *albumin* (*ALB*) gene. These mutant *ALB* molecules have higher affinity for thyroxine (T4) and reverse T3 (rT3), and to a lesser degree for triiodothyronine (T3). As a consequence, affected individuals have high serum total T4 (TT4) and rT3. The mutations all occur in amino acid 218 of the *ALB* molecule replacing the normal arginine with histidine, proline, or serine; the most common being histidine R218H (1). A gain-of-function mutations (L66P), with higher affinity to T3, has been reported in a single family (2) and another, R222I, with extremely high rT3 (3). Subjects harboring these *ALB* gene mutations are clinically euthyroid and thus have serum thyrotropin (TSH) values within the reference range.

FDH was first described in 1979 (4,5) and its cause, a mutation encoding the normal arginine-218 of the *albumin* (*ALB*) gene, was identified in 1994 independently in two laboratories (6,7). Despite clinical euthyroidism, patients are often misdiagnosed because of falsely elevated serum free T4 (fT4) concentration when measured by different immunoassays (8,9). In part, this is due to the interference of chloride in the buffer (10,11). Furthermore, different automated immunoassays yield variable results (12) and identifying an ideal condition to measure accurately fT4 in the presence of both wild-type and mutant *ALB* may prove to be difficult. We measured fT4 using direct dialysis coupled with tandem mass spectroscopy (fT4 DDMS) in individuals from 16 families

with FDH, 14 of whom had the common R218H mutation, and the results were compared with those obtained by a direct immunometric assay (fT4 DIMM) and by the free thyroxine index (fT4I). All individuals with *ALB* R218H gene mutation had elevated TT4 levels. An elevation of fT4 measured by DIMM was seen in affected individuals of 12 families, with the fT4I in affected individuals of 5 families, and by using DDMS in an affected individual of 1 family. The latter method greatly reduced the discordant results of fT4 in the common type of FDH.

Patients

Sera from 1 affected and 1 unaffected member of 16 families with FDH were included in the study. Fourteen families harbored the common *albumin* gene mutation (*ALB* R218H) causing FDH, and the other two families had either the *ALB* R218S (13) or the *ALB* R218P (14) gene mutations. Of the 43 families with *ALB* R218H studied in S.R.'s laboratory for the period of 25 years, sera from at least 1 affected and 1 unaffected individual were collected. Frozen samples from both affected and unaffected family members, which were collected at the same time, handled and stored in the same manner, and thawed no more than twice, were available from 16 families. Sample selection was based on matching as closely as possible an affected and an unaffected for sex and age. There were 15 samples from females and 17 from males and mean ages were 28.6 ± 4.4 and 32.6 ± 5.8 (SEM) years for affected and unaffected, respectively ($p=0.418$). Pairs were available from 1

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family with *ALB* R218S and 1 family with *ALB* R218P, both rare *ALB* gene mutations, and from 14 families with *ALB* R218H. All affected individuals were heterozygous.

Methods

The following tests were performed in a commercial laboratory (Quest Diagnostics Nichols Institute, San Juan Capistrano, CA). fT4 measurements by direct dialysis mass spectrometry (fT4 DDMS) were performed as briefly outlined hereunder. Samples (200 μ L) were dialyzed against an equal volume of a protein-free HEPES buffer (156 mM HEPES free acid, 90 mM NaCl, 5 mM urea, 3 mM Na₂HPO₄, 3 mM KCl, 2.5 mM CaCl₂·H₂O, 2 mM MgSO₄·7H₂O, and 8 mM NaN₃, pH adjust to 7.34 $\pm$ 0.01 using 10 N NaOH). We chose this dialysis buffer composition based on the recommendations in guideline C45A from the Clinical and Laboratory Standards Institute. The dialysis was performed for 17–19 hours at 37°C $\pm$ 1°C in a 96-well disposable dialysis plate (10k MWCO; Harvard Apparatus, Holliston, MA). The dialysate was then removed, combined with stable isotope labeled internal standard (T4-[¹³C₆]), and injected for high pressure chromatography and tandem mass spectrometry (HPLC-MS/MS)

analysis. The HPLC system used a Kinetex C18 2.6 μ m 4.6 $\times$ 50 mm stationary phase analytical column (Phenomenex, Torrance, CA) running an organic gradient employing 0.1% acetic acid and 100% methanol as mobile phases. T4 was detected using a tandem mass spectrometer (Quantiva; Thermo Fisher, Waltham, MA) using an heated electrospray ionization (HESI) interface with which HPLC flow is dispersed by electrospray into an aerosol, and the resulting fine liquid droplets are charged by the application of high voltage. Under the heated gas flow, solvent quickly evaporates leaving T4 and internal standard molecules with the charges. HESI was operated in the negative ion mode because more interference peaks and higher background were observed with the positive ion mode. Acetic acid concentration (0.1%) in the mobile phase was optimized for maximum and consistent signal intensity. T4 and the T4-[¹³C₆] internal standard were detected and quantitated using multiple-reaction-monitoring (MRM). MRM ion pairs 775.7 $\rightarrow$ 126.9 and 781.7 $\rightarrow$ 126.9 were used for T4 and internal standard, respectively. Concentrations of fT4 in the dialysate were determined using peak area ratio and a calibration curve (0.2–12.8 ng/dL). The limit of quantitation for this assay was determined to be 0.2 ng/dL and the analytical measuring range was 0.2–12.8 ng/dL. The intra-assay and

TABLE 1. DEMOGRAPHICS AND THYROID TEST RESULTS IN ALL STUDIED INDIVIDUALS

Family no.	Age (years)	Sex	TSH (mU/L)	Total T4 (μ g/dL)	fT4I	fT4 DDMS (ng/dL)	fT4 DIMM (ng/dL)	Mutant albumin (+) ^a
67	59	M	2.4	18.3	12.4	2.0	2.9	+
67	55	F	2.4	8.3	8.4	1.5	1.2	–
66	9	F	3.2	18.7	9.1	2.0	1.9	+
66	26	F	1.9	8.4	9.1	1.6	1.3	–
65	35	M	2.1	17.2	12.3	2.2	2.8	+
65	49	M	2.3	7.4	9.6	2.4	1.5	–
64a	53	M	1.8	21.5	9.5	2.0	3.0	+
64a	50	F	2.7	8.9	9.2	1.6	1.1	–
64b	48	M	1.7	15.3	11.3	2.2	2.7	+
64b	48	F	3.2	10.4	9.7	1.9	1.5	–
64c	24	F	3.4	16.7	7.7	2.0	2.3	+
64c	58	F	3.3	7.0	7.1	1.4	1.1	–
62	60	F	2.0	12.2	8.8	1.6	1.8	+
62	60	M	2.9	9.8	9.9	2.2	1.3	–
61	40	F	2.6	14.5	9.0	1.7	1.8	+
61	43	M	1.8	8.6	9.4	1.6	1.3	–
59	74	M	3.7	14.9	8.0	1.5	1.9	+
59	35	M	2.8	7.6	8.5	1.7	1.5	–
49	4.8	F	4.1	20.9	7.9	2.2	2.9	+
49	2	F	1.9	8.4	9.6	2.1	1.5	–
58	10	M	1.4	18.7	13.1	2.1	2.4	+
58	8	M	1.7	8.8	8.4	1.9	1.2	–
47	1.5	F	2.2	16.4	12.0	1.7	2.4	+
47	6	F	0.7	8.5	9.9	1.8	1.4	–
37	0.5	M	1.7	18.5	19.8	2.9	3.6	+
37	35	M	0.9	10.3	10.0	2.3	1.5	–
33	48	M	1.9	15.1	10.4	1.7	1.9	+
33	47	M	1.4	8.0	7.7	1.6	1.1	–
57	25	F	3.8	85.0	33.0	1.9	7.0	+ R218S
57	34	F	3.8	9.1	10.4	2.1	1.6	–
43	29	M	1.6	116	133	9.0	QNS	+ R218P
43	29	M	1.0	7.7	9.5	2.6	1.5	–

Bold numbers are above the normal range for the test and age. “a,” “b,” and “c” are nuclear families of the same large family.

“+” without indication of the mutation are individuals with albumin R218H; “–” relative without mutation.

fT4, free thyroxine; DDMS, direct dialysis coupled with tandem mass spectrometry; DIMM, direct immunometric assay; fT4I, free thyroxine index; TSH, thyrotropin; QNS, quantity not sufficient.

TABLE 2. COMPARISON OF VALUES (MEAN $\pm$ SEM) OF AFFECTED AND UNAFFECTED INDIVIDUALS

Test	R218H (n = 14)	WT (n = 14)	p	
			Unpaired	Paired
fT4I	10.7 $\pm$ 0.91	9.02 $\pm$ 0.26	0.101	0.08
fT4 MS (ng/dL)	1.99 $\pm$ 0.10	1.86 $\pm$ 0.09	0.374	0.113
fT4 DIMM (ng/dL)	2.45 $\pm$ 0.15	1.32 $\pm$ 0.05	<0.0001	<0.0001
TT4	17.1 $\pm$ 0.69	8.61 $\pm$ 0.29	<0.0001	<0.0001
TSH (mU/L)	2.44 $\pm$ 0.22	2.25 $\pm$ 0.20	0.521	0.285
Age (years)	33.3 $\pm$ 6.6	39.7 $\pm$ 5.0	0.459	0.427

WT, wild-type.

interassay variation ranged from 7.1% to 9.1% and from 7.3% to 9.4%, respectively, over a concentration range of 0.39–2.24 ng/dL. No significant interference was observed except with grossly lipemic samples. T3 could be chromatographically separated (with a retention time of $\sim$ 0.4 min earlier) using MRM ion pair of 649.7 $\rightarrow$ 126.9. Comparison with a reference method offered at national reference laboratory at the University of Utah (7) was conducted using 82 split patient samples. Correlation analysis between the 2 sets of data showed a slope of 0.961, an intercept of 0.13 ng/dL, and correlation coefficient of 0.991.

Reference intervals for DDMS employed several different subject groups. Sample collection was approved by an IRB-consented protocol. For adults, the reference population comprised 132 (56 females and 76 males) normal, apparently healthy, ambulatory, community dwelling, and nonmedicated adults employed at Quest Diagnostics Nichols Institute between the ages of 18–64 years. Individuals with an abnormal TSH or a history of thyroid disease were excluded. No sex difference was evident and a single adult reference interval based on the mean $\pm$ 2 SD range was derived, 0.9–2.2 ng/mL. For pediatric subjects, remnant samples submitted to San Juan Capistrano for either acylcarnitine or insulin-like growth factor-1 (IGF-1) testing were employed. All samples were stored frozen (-70°C) for 21 days until they had passed their required retention time. After deidentification, 103 subjects (50 females and 53 males) between the ages of 8 days and 2 years and 164 (105 females and 59 males) between the ages of 3 and 17 years were tested. The criteria for inclusion required either IGF-1 result (for samples submitted for IGF-1) or acylcarnitine result (for samples submitted for acylcarnitine) within the reference range. All samples were screened to confirm a TSH in the reference range before consideration for testing for inclusion in the reference interval. Reference intervals were prepared on Box-Cox transformed data assuming a Gaussian distribution is cited for the 8-day to 2-year age group, 1.2–2.5 ng/dL. Reference intervals based on the percentile method are cited for the 3–17-year age group, 1.0–2.4 ng/dL. The percentile-based method was cited for the combined analysis. The resulting reference intervals for the direct dialysis, HPLC method, overlapped with the existing reference intervals of the predicate method.

The fT4 measured by direct immunometric assay (fT4 DIMM) utilized Centaur (Siemens, Tarrytown, NY). It is a competitive immunoassay using chemiluminescent technology. fT4 in the specimen competes with acridinium ester labeled T4 for a limited amount of biotinylated polyclonal rabbit anti-T4 antibody. The biotinylated anti-T4 binds to

avidin that is covalently coupled to paramagnetic particles. After incubation, the particles are washed to remove any unbound fT4 and tracer and the bound chemiluminescence is quantitated in relative light units. The fT4 concentration is calculated by comparison with a standard curve. The intra- and interassay coefficients of variation for this assay varied from 1.9% to 6.5% and from 4.1% to 6.5%, respectively. Reference ranges for fT4 DIMM (ng/dL) are as follows: 0.8–1.4 for 0.1–12 years, 0.8–1.4 for 13–20 years, and 0.8–1.8 for >20 years.

The following tests were carried out in the University of Chicago laboratory. fT4I was determined from the product of the TT4 and the resin-T4 uptake ratio. The latter was performed in 75 mM barbital buffer, pH 8.6 (15), the reference

FIG. 1. Z-test score comparison for fT4 measurement methods in albumin R218H mutation. Z-test score = (fT4 value – mean of fT4 in the albumin wild-type group)/standard deviation of the albumin wild-type group. “–” denotes individuals without FDH. “+” refers to individuals with FDH caused by the albumin R218H. The horizontal dashed lines denote the $\pm$ 2 SD ranges calculated using results from the 14 individual without FDH. In the fT4 DDMS method, all in FDH group except one were within the mean $\pm$ 2 SD range. Six out of 14 were above the corresponding wild-type group range with fT4I method. All the results in the FDH group were above the wild-type range using the fT4 DIMM method. DDMS, direct dialysis coupled with tandem mass spectrometry; DIMM, direct immunometric assay; FDH, familial dysalbuminemic hyperthyroxinemia; fT4, free T4; fT4I, free thyroxine index; SD, standard deviation.

range for all ages was 6.0–10.5. The TT4 was measured by Elecsys (Roche, Indianapolis, IN), the reference range for all ages was 5.0–12.0 $\mu\text{g/dL}$. The resin-T4 uptake used an in-house method in which ^{125}I -labeled T4 is added to the serum and the partition of radioactivity between resin (Amberlite IRA 400 Chloride; Aldrich Chemistry, Atlanta, GA) and that remaining in solution in the samples divided by the same in a pool of serum from healthy individuals without thyroid pathology represented the resin-T4 uptake ratio.

Results

All affected individuals had TT4 above and unaffected individuals had TT4 within the reference range. In contrast, TSH (Elecsys by Roche) was within the reference range for age in all affected and unaffected individuals and was not significantly different for age (Table 1). The mean age of affected (38.6 ± 4.4 years) and unaffected (32.6 ± 5.8 years) was not significantly different ($p = 0.418$).

Analysis of data from the families with ALB R218H showed significant differences between affected and unaffected subjects in TT4, and fT4 DIMM, but not fT4I and fT4 DDMS (Table 2). Importantly, of the 14 affected individuals, fT4 DIMM was higher than the quoted 95% reference interval in 12, fT4I in 5, and fT4 DDMS in 1 individual. In addition fT4 DIMM was slightly elevated in one unaffected 2-year old (Table 1). To compare those three methods of fT4 measurement in individuals with FDH, the box-and-whisker plot of the Z score was generated (Fig. 1). The DDMS method demonstrated almost no difference between the control and the FDH group, with only one result above the control group in the FDH group. The fT4I method showed moderate performance with 6 out of 14 above the control group. DIMM was the least favorable method as all the results from the FDH group were significantly higher than its control group.

Passing-Bablok linear regression was generated to further compare DIMM with DDMS method (Fig. 2). The DIMM method compared with the DDMS method had a positive bias in the FDH group. However, it exhibited negative bias compared with the DDMS method in the control group.

The albumin of individuals expressing ALB R218S and R218P has very high affinity for T4, resulting in TT4 values of 85 and 116 $\mu\text{g/dL}$, or 10.6- and 14.5-fold the normal mean, respectively. The fT4 DDMS measurement was within the reference range in the only affected expressing ALB R218S. Measurements of fT4 by all other methods gave high values, as did all fT4 tests of the subject with ALB R218P (Table 1).

Discussion

It is known that standard direct immunoassays for fT4 produce falsely high values in individuals with FDH (8,12). This is due, in part, to the concentration of chloride in the buffer by inhibiting T4 binding to albumin (10,11). The effect is much stronger in sera containing ALB R218H, possibly because of higher amounts of T4 bound to the mutant protein (9–11). However, other causes may intervene, such as the method, as elevated fT4 is observed in some subjects with FDH when this entity is estimated by the fT4I measured in barbital buffer with no chloride. The purpose of this study was to assess the performance of a newly developed test that couples direct dialysis with determination of T4 by tandem mass spectrometry (fT4 DDMS) in subjects with FDH and

FIG. 2. Passing-Bablok regression of fT4 DDMS versus DIMM in individuals without (open circle) and with (solid circle) FDH caused by the albumin R218H. The X-axis represents fT4 values by DIMM (ng/dL). The Y-axis represents fT4 values by DDMS (ng/dL). The solid line represents the Passing-Bablok regression line of 14 individuals with FDH who harbor albumin R218H. The dashed line (— — —) represents the Passing-Bablok regression line of 14 matched individuals without FDH (2 pairs have the same values, see Table 1 for the raw values). The dotted line (.....) represents the identity line.

compare the results with those using an automated immunometric fT4 DIMM, as well as the fT4 estimate using measurement of TT4 with a resin-T4 uptake (fT4I). Blood samples were derived from individuals referred to the laboratory of S.R. because of discrepant thyroid test results, mostly high fT4 with unsuppressed TSH. A protocol approved by the IRB required the collection of samples from members of the nuclear family for further testing, which included sequencing of the *ALB* gene. The reason for selecting a single sample of an affected and an unaffected member from a different nuclear family in this study is based on the fact that, for unknown reasons, the magnitude of TT4 elevation varies among families harboring the same *ALB* gene mutation.

Results show that fT4 DDMS outperformed the fT4I and fT4 DIMM with only 1 subject having values above the reference range compared with 5 and 12 using the other 2 methods, respectively. The fT4 DDMS was within the reference range for the affected individual with ALB R218S, whose TT4 was 85 $\mu\text{g/dL}$, but not for the individual with ALB R218P, whose TT4 was 116 $\mu\text{g/dL}$. This is likely due to the dialysis conditions for an albumin variant with extremely high affinity for T4 (14). The other two methods gave spurious results in both affected individuals.

Disclaimer

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