Annals of Thyroid Research

Open Access

Austin Publishing Group

Research Article

Comparison of Thyroid Panel by Immunoassay and Liquid Chromatography-Tandem Mass Spectrometry during Transition from Euthyroid to Hyperthyroid State

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Received: December 19, 2018; **Accepted:** February 01, 2019; **Published:** February 08, 2019

Abstract

Accurate measurement of thyroid hormones (TH) is critical for thyroid disease management. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) was shown to be superior to Immunoassay (IA) in states of hypothyroidism and protein binding interference. The study aims were to compare THs measured by IA *vs* LC-MS/MS in the hyperthyroid state, and investigate the correlation of THs by IA *vs* LC-MS/MS with central and peripheral markers of TH action.

Materials and Methods: Six patients were enrolled in a study assessing effects of Triiodothyronine (T3) on non-insulin mediated glucose disposal. TSH, fT4, TT4, fT3, TT3, and TBG were checked at baseline and daily for 2 weeks by IA and LC-MS/MS. Peripheral markers of TH action were measured at baseline and weekly.

Results: Concordance between IA and LC-MS/MS for fT4, TT4, fT3, and TT3 varied (estimation for slope 0.44, 0.63, 0.32, and 0.7, respectively, $p<0.0001$ for all). TSH was significantly associated with TT3 and fT3 by both IA (TT3 p=0.017, fT3 p=0.02) and LC-MS/MS (TT3 p=0.001, fT3 p=0.023) with no difference between methods. Lipids were significantly associated with TT3 by IA (HDL p=0.009, LDL p=0.02) and LC-MS/MS (HDL p=0.01, LDL p=0.002); fT3 was significantly associated with HDL (p=0.01) and LDL (p=0.01) only by IA, but there was no difference between methods.

Conclusion: TH measured by IA and LC-MS/MS were significantly correlated with one another throughout the transition from euthyroid to hyperthyroid state. IA and LC-MS/MS were equally valid to evaluate hyperthyroidism. However, larger studies are needed to validate these findings.

Keywords: Hyperthyroidism; Immunoassay; Liquid chromatography-tandem mass spectrometry

Introduction

Thyroid dysfunction, including both hyper- and hypothyroidism, is prevalent in clinical practice. The quality of thyroid hormone measurement is critical for accurate diagnosis since the signs and symptoms of thyroid diseases often are nonspecific, subtle, or absent. Thyroid stimulating hormone (TSH) is currently considered to be the most sensitive screening test for initial assessment of hypothalamic-pituitary-thyroid axis (HPT) function, because TSH secretion by the pituitary gland responds robustly to even slight changes in levels of free thyroid hormones [1]. TSH is widely available, safe and inexpensive. However, the use of TSH as the best single or initial test for hyper- and hypothyroidism is limited by inter-individual variation, diurnal fluctuations, gender-related differences, age-related changes in TSH, primary pituitary disorders, anti-animal antibodies interfering with assays, and medications that alter TSH secretion [2-9]. Metabolic markers of thyroid hormone action (e.g. lipids or sex hormone binding globulin) are not recommended for routine clinical assessment of thyroid status since these parameters are not sensitive, specific, or standardized [10].

Measurement of thyroid hormones (T3, T4) provides important information about thyroid function beyond TSH, especially in contexts in which TSH may provide erroneous information, as described above. The accepted reference method for measurement of fT4 are equilibrium dialysis or ultrafiltration (UF) of serum [11]. Because measurement of T4 by equilibrium dialysis is time-consuming, labor intensive, technically demanding, and expensive, the most common method for TH measurement is direct analog immunoassay (IA) [12]. However, this technique is limited by multiple factors [12].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is an improved alternative to IA for measurement of thyroid hormones, and is the current reference method technique [13,14]. LC-MS/MS is superior to immunoassay for detection of thyroid hormone concentrations in hospitalized patients, patients with hypothyroidism, protein-binding abnormalities, non-thyroidal illness, and in patients taking multiple medications [13,15,16]. Furthermore, T3 and T4 measured by LC-MS/MS were shown to correlate better with log TSH values compared to T3 and T4 concentrations measured by IA [17]. However, only a single study included measurement of thyroid

Citation: Kushchayeva Y, Soldin SJ, Stolze B, Yu X, Auh S, Lin TC, et al. Comparison of Thyroid Panel by Immunoassay and Liquid Chromatography-Tandem Mass Spectrometry during Transition from Euthyroid to Hyperthyroid State. Annals Thyroid Res. 2019; 5(1): 178-184.

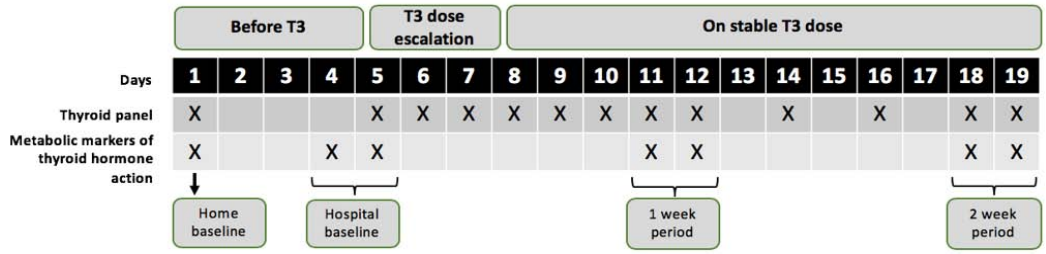

Figure 1: Euthyroid patients with mutations of the insulin receptor were studied for 5 days prior to T3, underwent 3 days of T3 dose escalation to achieve a mild hyperthyroid state, and were maintained on T3 for a total of 14 days. Thyroid panel included TSH, fT4, TT4, fT3, TT3, and TBG. Free T4, total T4, free T3 and total T3 were measured by both immunoassay and LC-MS/MS. Metabolic markers of thyroid hormone action included total cholesterol, HDL-c, LDL-c, triglycerides, sex hormone binding globulin, free fatty acids, and osteocalcin.

hormones by IA and LC-MS/MS in hyperthyroid individuals [11] and validity was assessed based on correlations with TSH levels, only.

The study goal was to compare measurement of thyroid hormones by IA *versus* LC-MS/MS during transition from the euthyroid to the hyperthyroid state in a cohort of patients, and to determine which technique correlates better with TSH and peripheral metabolic markers of thyroid hormone action.

Materials and Methods

Patients and study design

Six patients with mutation in the insulin receptor gene (INSR) (either recessive or dominant negative), extreme insulin resistance, and diabetes mellitus were enrolled in a study of liothyronine (T3, the active form of thyroid hormone) to increase non-insulin-mediated glucose disposal (NCT02457897). The current study comparing assays for thyroid hormones was an ancillary study. Eligible patients were aged 12 to 65 years, on stable doses of home medications for the preceding 10 weeks, euthyroid, and had no history of medical conditions or medications that would increase risk of liothyronine use or alter measurement or absorption of thyroid hormones. The study was approved by the NIDDK Institutional Review Board. All patients or their guardians provided written informed consent prior to participation, and minors provided written assent. The study design is shown in Figure 1.

For analyses, "home baseline" levels were defined as levels measured the morning after admission and "hospital baseline" as the mean of measurements obtained on hospital days 4 and 5 before liothyronine treatment was initiated.

All patients were euthyroid at baseline with initial evaluation in the euthyroid state for four days before liothyronine treatment. Liothyronine was then initiated at 0.57 mcg/kg/day divided every eight hours on day 5, 0.885 mcg/kg/day on day 6, and 1.14 mcg/kg/day on day 7. Thereafter, the liothyronine dose was titrated to achieve T3 peak that was 25-50% above the upper normal limit of 200 ng/dL (i.e. 250-300 ng/dL) measured 3 hours after the morning liothyronine dose and T3 trough target of 150-250 ng/dL measured immediately prior to the morning liothyronine dose. Peak and trough levels were measured daily for the first week, and every other day thereafter unless further dose adjustment was needed.

Plasma TSH, fT4, TT4, fT3, TT3, and TBG were measured at home baseline and hospital baseline, daily during the first week on treatment, and every other day for the next week. Samples were obtained immediately prior to the morning dose of liothyronine during the liothyronine treatment period. Peripheral markers of TH action (lipids, osteocalcin, FFA, and SHBG) were measured at home baseline (day 1), hospital baseline (days 4 and 5), one week (days 11 and 12) and 2 weeks (days 18 and 19) on liothyronine.

Laboratory methods

Blood samples for measurement of serum thyroid hormone levels were collected in lithium heparin tubes. Measurement of thyroid hormones by both IA and LC-MS/MS were performed using the same blood samples at each time point. TSH, fT4, TT4, fT3, and TT3 levels measured by IA were analyzed on the same day the blood was drawn using the Roche Cobas 6000 (Indianapolis, IN) analyzer. IA thyroid hormone reference intervals for TSH, fT3, fT4, TT3, and TT4 (0.27-4.20 mcIU/ml, 2.0-4.4 pg/mL, 0.9-1.7 ng/dL, 80-200 ng/dL, and 4.5-11.7 µg/dL) were suggested by the manufacturer.

For the LC-MS/MS method, samples for fT3, TT3, fT4 and TT4 measurement were stored at −80 degrees Celsius until analysis was performed. Measurement of fT3 and fT4 was done by ultrafiltration isotope dilution LC-MS/MS using a SCIEX Triple-Quad-6500 System (Framingham, MA) as described by van Deventer [11,15], with complete method validation details previously published [11,18]. Briefly, 400 µL of plasma was filtered through a Centrifree ultrafiltration device at 37˚C. Two-hundred fifty microliters of internal standard (T4-13C6, T3-13C6) in methanol was then added to 150 µL of ultrafiltrate for deproteinization, and 325 µL of supernatant was diluted into 675 µL of deionized water and a 400 µL aliquot was injected onto a Poroshell 120 EC-C18 column. Quantification by multiple reaction monitoring analysis was performed in the negative mode. Recoveries for fT3 and fT4 were between 95 and 105% and the intra- and inter-assay coefficients of variation were 9% for fT3 and 7% for fT4. TT3, TT4, and rT3 were assayed by LC-MS/MS using an Agilent 6460-Triple-Quad System as previously described [19]. Briefly, 100 µL of sample was added to 150 µL of 13˚C labeled internal standard for deproteinization. Then 200 µL of supernatant was diluted into 500 µL of 0.1 M ammonium acetate in deionized water and 200 µL injected onto an Agilent Eclipse XBD-C8 cartridge column, eluted with a water/methanol gradient containing 0.01% formic acid into the MS/MS system. Quantification by multiple reaction mode monitoring was performed in the positive mode. Method validation details have been previously described [19,20]; recovery ranged from 92.8% to 95.4% and the intra-assay coefficient

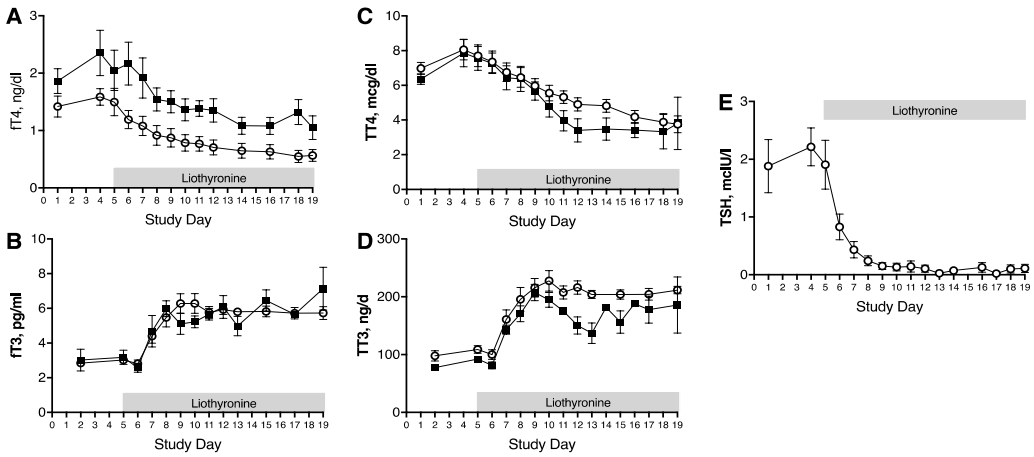

Figure 2: A) Free T4 (fT4), B) free T3 (fT3), C) total T4 (TT4), D) total T3 (TT3), and E) thyroid stimulating hormone (TSH) before and during two weeks of liothyronine treatment measured by immunoassay (open circles) and LC-MS/MS (black squares). The gray shaded areas indicate the period of liothyronine treatment (starting on day 5). Data represented as mean±SEM.

of variation was between 1.6% and 7.6%. LC-MS/MS plasma thyroid hormone reference intervals were 2.2-6.2 pg/mL for FT3, 1.3-2.4 ng/dL for FT4, 80-177 ng/dL for TT3, 5.0-10.9 µg/dL for TT4, and 9-24 ng/dL for rT3 [18,21].

TBG was measured on the Siemens Immulite 2000 XPI analyzer (Malvern, PA) and albumin by the Roche Cobas 6000 (Indianapolis, IN).

Statistical methods

Variables with skewed distribution were Box-Cox transformed prior to analysis.

To account for repeated measures for each patient, linear mixed models with multiple comparisons for multiple time points were applied to compare fT4, TT4, fT3, TT3 and peripheral markers of TH action at home baseline (day 1) or hospital baseline (days 4 and 5), the end of the first week (days 11 and 12) and the end of the second week (days 18 and 19) after starting T3 treatment. If the T3 status effect was significant, post-hoc multiple comparisons analyses were conducted to test the difference between each pair of time points.

The linear mixed model was applied to study the effect of each independent variable of TSH, rT3, and the lipid panel variables for each dependent variable of TT3, TT4, fT3, and fT4. First, an estimate of parameter for each independent variable on each dependent variable was obtained for each method respectively. Second, additional terms of the measuring method (either IA or LC-MS/MS) and the interaction between the independent variable and the measuring method were included into the first linear mixed model in order to evaluate whether the association between the independent and dependent variables was different between the two methods. Significant interaction effects indicate that the relationship between the independent and dependent variables differed between the IA and LC-MS/MS methods. A p-value <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA) and SAS software version 9.4 (SAS Institute Inc., Vary, NC, USA).

Results

Patients characteristics

The mean age of patients was 22.2 ± 6.5 years. There were three males and three females. All patients had proven INSR mutations.

Thyroid status before and during liothyronine treatment

Thyroid hormone levels (TSH, fT4, fT3, TT4, and TT3) were not different at home baseline *versus* hospital baseline by either IA or MS (p>0.05).

As expected, TSH, fT4, TT4, and rT3 significantly decreased and fT3 and TT3 significantly increased after 2 weeks of liothyronine treatment measured by both immunoassay (TSH was measured by immunoassay only) and LC-MS/MS (rT3 was measured by LC-MS/MS only) (Table 1, Figure 2).

TSH at hospital baseline (day 5, immediately before the first dose of liothyronine) was 1.9±0.4 mcIU/ml, and decreased significantly to 0.8±0.2 mcIU/ml (p<0.05) on day 6 after one day of liothyronine treatment. Stable TSH levels below the normal range (<0.27 mcIU/ml) were achieved by day 8 (three days of liothyronine treatment) (Figure 2). Free T3 and TT3 increased to the hyperthyroid range by study days 7 and 8, respectively. Target TT3 trough (150-250 ng/dl) was achieved on day 6 (after only 3 doses of liothyronine) (160.8±16.5 ng/dl), was above 200 ng/dl on day 8, and stayed at target during 2 weeks of liothyronine treatment. TT3 peak levels increased from 160.0±12.0 ng/dl after the first dose to 247.7±30.0 ng/dl on day 6, and remained stable above 250 ng/dlon day 7 and thereafter during liothyronine treatment.

Free T4 was 1.4±0.1 ng/dl at hospital baseline, and decreased significantly by day 8 to 1.1±0.1 ng/dl (p=0.045). TT4 significantly decreased from 7.7±0.6 mcg/dl at hospital baseline to 6.0±0.4 mcg/dl by day 9 on liothyronine (p=0.002). Reverse T3 (rT3) significantly decreased from 25.5±4.0 ng/dl at hospital baseline to 7.4±1.7 ng/dl after one week (p=0.0086) and 8.2±3.0 after 2 weeks of liothyronine (p=0.0032).

Thyroxin-binding globulin (TBG) and albumin did not change

Table 1: Thyroid panel measured by IA and LC-MS/MS before and during liothyronine treatment.

Thyroid panel, plasma	Hospital baseline	2 weeks on T3	Reference intervals	P-value
Immunoassay				
TSH, mcIU/ml	2.06±0.26	0.1±0.04	0.27-4.20	<0.001
fT4, ng/dl	1.44±0.09	0.66±0.065	0.9-1.70	<0.001
TT4, ng/dl	7.875±0.4	3.8±0.33	4.4-12.2	0.001
fT3, pg/ml	2.9±0.45	5.63±0.7	2.0-4.4	<0.001
TT3, ng/dl	104.4±18.1	206.96±18	80-200	<0.001
LC-MS/MS				
rT3, ng/dl	24.9±3.2	9.2±2.8	21-Sep	<0.01
fT4, ng/dl	2.2±0.25	1.2±0.15	1.3-2.4	<0.05
TT4, ng/dl	7.69±0.5	3.5±0.79	5.1-11	<0.05
fT3, pg/ml	2.88±0.25	5.48±0.36	1.5-6.2	<0.001
TT3, ng/dl	86.36±4.3	196±33.7	80-187	0.001

Data represented as mean±SEM; LC-MS/MS; Liquid Chromatography-Tandem Mass Spectrometry

Table 2: Association of fT3 and TT3 measured by IA and LC-MS/MS with peripheral markers of TH action and comparison of IA and LC-MS/MS.

Method	Dependent variable	Independent variable	Estimation for slope	p value for association	Method difference on association, IA *vs* LC-MS/MS, p-value
IA	TT3	Total Cholesterol	-0.216	0.221	0.602
LC-MS/MS	TT3	Total Cholesterol	-0.462	0.598	
IA	fT3	Total Cholesterol	-0.005	0.268	0.588
LC-MS/MS	fT3	Total Cholesterol	-0.002	0.812	
IA	TT3	LDL-c	-0.913	0.017	0.197
LC-MS/MS	TT3	LDL-c	-1.605	0.002	
IA	fT3	LDL-c	-0.027	0.01	0.899
LC-MS/MS	fT3	LDL-c	-0.025	0.08	
IA	TT3	HDL-c	-1.067	0.009	0.581
LC-MS/MS	TT3	HDL-c	-1.725	0.013	
IA	fT3	HDL-c	-0.024	0.035	0.392
LC-MS/MS	fT3	HDL-c	-0.016	0.378	
IA	TT3	Osteocalcin	0.175	0.065	0.549
LC-MS/MS	TT3	Osteocalcin	1.074	0.096	
IA	fT3	Osteocalcin	0.004	0.684	0.566
LC-MS/MS	fT3	Osteocalcin	0.013	0.333	
IA	TT3	FFA	54.18	0.208	0.907
LC-MS/MS	TT3	FFA	68.89	0.467	
IA	fT3	FFA	1.269	0.28	0.747
LC-MS/MS	fT3	FFA	0.945	0.604	
IA	TT3	SHBG	-0.34	0.139	0.794
LC-MS/MS	TT3	SHBG	-0.58	0.026	
IA	fT3	SHBG	-0.008	0.205	0.869
LC-MS/MS	fT3	SHBG	-0.006	0.469	

IA: Immunoassay; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; HDL-c; High Density Lipoprotein Cholesterol; LDL-c; Low Density Lipoprotein Cholesterol; FFA; Free Fatty Acids; SHBG; Sex Hormone Binding Globulin

during T3 treatment. Mean albumin was 3.9±0.05 mcg/ml before and 3.9±0.1 mcg/ml after 2 weeks on liothyronine. TBG was 6.1±1.2 mcg/ml at hospital baseline and 16.6±1.6 mcg/dl after 2 weeks of liothyronine.

Association between thyroid hormones measured by immunoassay and LC-MS/MS

T4, fT4, TT3 and fT3 measured by IA strongly correlated with measurements by LC-MS/MS ($p<0.0001$ for all) with estimates for

slope of 0.74 for TT3, 0.63 for TT4, 0.32 for fT3, and 0.45 for fT4.

Association between thyroid panel variables and TSH

TSH significantly correlated with TT3 and fT3 measured by both IA (TT3 p=0.03, fT3 p=0.034) and LC-MS/MS (TT3 p=0.028, fT3 p=0.036). The association between thyroid hormones and TSH did not differ between IA and LC-MS/MS methods (p>0.05).

Effect of thyroid hormone excess on peripheral metabolic markers of TH action (lipids, SHBG, osteocalcin, and FFAs)

Total cholesterol decreased significantly from 142.0±7.0 mg/dl at hospital baseline to 127.0±4.0 mg/dl and 121.6±3.0 mg/dl after one and two weeks of liothyronine, respectively (p=0.03 and 0.016, respectively, *versus* hospital baseline). LDL decreased from 71.0±5.0 mg/dl at hospital baseline to 59.5±5.0 mg/dl and 58.0±5.0 mg/dl after one and two weeks of liothyronine (p=0.024 and 0.039, respectively, *versus* hospital baseline).

HDL-c decreased from 61.5±4.0 g/dl at hospital baseline to 57.5±5.0 mg/dl and 54.0±5.0 mg/dl after one and two weeks of liothyronine (p=0.4 and 0.04, respectively, *versus* hospital baseline).

FFAs did not change during liothyronine treatment (0.6±0.1 mmol/L at baseline, 0.5±0.1 mmol/L after 1 week, 0.6±0.1 mmol/L after 2 weeks, p>0.05). Osteocalcin did not change before and after liothyronine (37±13 ng/ml at hospital baseline, 37±11 ng/ml after 1 week, 40±13 ng/ml after 2 weeks, p>0.05). SHBG did not change before and after liothyronine (65±17 mg/dl at hospital baseline, 72±20 mg/dl after 1 week, 71±20 mg/dl after two weeks, p>0.05).

Association of thyroid hormone levels with metabolic markers of thyroid hormone action (lipids, SHBG, osteocalcin, and FFAs) by IA and LC-MS/MS methods

LDL-c was significantly associated with fT3 by IA (p=0.01) but did not reach significance by LC-MS/MS (p=0.08). LDL-c was significantly associated with TT3 by both IA and LC-MS/MS (p=0.02 and 0.002, respectively). HDL-c was significantly associated with fT3 and TT3 measured by IA (p=0.035 and 0.009) and with TT3 by LC-MS/MS (p=0.01). No association of SHBG, osteocalcin, or FFAs with either TT3 or fT3 was found by either method (Table 3). No significant difference between IA or LC-MS/MS methods for association of any thyroid hormone with metabolic markers of thyroid hormone action was found on linear mixed model analyses (Table 2).

Discussion

This is the first study comparing measurement of thyroid hormonesby IA *versus* LC-MS/MS during transition from the euthyroid to the hyperthyroid state in a single cohort of patients, thus avoiding confounding effects of inter-individual variation. We demonstrated high agreement in thyroid hormone levels between the two techniques. Furthermore, both techniques correlated similarly with TSH and metabolic markers of thyroid hormone action in both the euthyroid and the hyperthyroid state.

Currently most clinical laboratories use automated IA platforms for detection of TH levels. However, the validity of free thyroid hormone analysis by direct analog immunoassay has been questioned [16,22]. Multiple factors including abnormal protein binding, dialyzable protein binding competitors, heterophile antibodies, autoantibodies, high binding protein levels (e.g. during critical illness or pregnancy), and *in vitro* factors such as FFAs, biotin, some drugs, assay antibodies, analogs, and assay dilution steps can affect the IA results [11,23,24].

The standard for measurement of thyroid hormones is Nichols equilibrium dialysis and ultrafiltration, in which the free analyte is separated from proteins prior to measurement followed by IA [11]. However, the equilibrium dialysis technique is labor intensive, imprecise, technically demanding, time consuming (17-24 h to reach an equilibrium), temperature and pH dependent, expensive, and is not available in most clinical laboratories [12]. Ultrafiltration is also temperature dependent but requires less time (30 minutes) and was shown to have excellent correlation with equilibrium dialysis in fT4 measurement at 37˚C [17].

LC-MS/MS is an alternative to traditional ligand-binding assays for the quantitative determination of analytes of interest. LC-MS/MS has already revolutionized measurement of steroid hormones, resulting in dramatic improvements in sensitivity and specificity for multiple endocrine tests including aldosterone, cortisol, cortisone, estrogens, and testosterone [25]. Advantages of LC-MS/MS are small sample volume, measurement of hormones in relatively low concentrations, fast analysis times, and the possibility of simultaneous measurement of many analytes [26]. Although MS assays are not more precise then IA, they are more specific for analytes of interest [27]. However, MS methods are labor intensive, require high level of expertise, have high instrument costs, greater technical complexity, and thus are not practical for rapid analysis of large numbers of patient samples. LC-MS/MS methods also require the removal of protein and preferably other interfering substances from the sample before analysis, as proteins can cause instrument blockages with deleterious effects on the analysis.

The measurement of THs by LC-MS/MS has been already described, and comparison of UF LC-MS/MS with a reference method, equilibrium dialysis, showed identical results for quantification of free THs [13,14]. The correlation between fT4 and log-transformed TSH was superior in LC-MS/MS *versus* IA in pediatric, healthy adult, pregnant and non-pregnant subjects [17]. In patients with hypothyroidism IA has been shown to overestimate values for fT3, TT3, and fT4 at low concentrations compared to LC-MS/MS [22], likely due to the presence of thyroid binding proteins. LC-MS/MS overcomes this problem by removal of proteins prior to analysis [22].

In agreement with previous studies we found that TT3 and TT4 were lower when measured by LC-MS/MS *versus* IA [23], and better agreement between methods for total thyroid hormones (TT3 and TT4; slope 0.74 and 0.63) compared to free thyroid hormones (fT3 and fT4; slope 0.32 and 0.45) [17,28]. We speculate that better agreement between methods for measurement of total *versus* free thyroid hormones might be because free hormones are measured in picomolar ranges and LC-MS/MS has greater sensitivity for low analyte concentrations. Another explanation might be the presence of thyroid binding proteins that interfere with free hormone analyses by IA; because these binding proteins are removed prior to LC-MS/MS this could lead to discrepancies between methods.

To better understand the physiologic relevance of THs measured by IA *versus* LC-MS/MS, we analyzed the correlation of THs by both

methods to both central (TSH) and peripheral (lipids, osteocalcin, SHBG, FFA) markers of thyroid hormone action. Previously, fT3 measured by LC-MS/MS has been demonstrated to have better correlation with TSH in comparison to IA (r=-0.72 *vs* r=-0.57) [17]. We found a significant association of TSH with fT3 and TT3 using both IA and LC-MS/MS without statistical differences between methods. A prior study showed better correlation of fT4 with TSH measured by LC-MS/MS (r=-0.59) *vs* IA (r=-0.48) [17]. In the current study, we did not test the association of fT4 and TT4 with TSH because production of endogenous fT4 and TT4 was suppressed by exogenous T3 (liothyronine) administration. As expected, thyroid hormones correlated with metabolic markers of thyroid hormone action. However, the strength of these correlations did not differ depending on the method for thyroid hormone measurement (IA *vs* LC-MS/MS).

LC-MS/MS has greater sensitivity compared to IA to detect mild hypothyroidism. As many as 45% of patients with normal TT3 by multiple different automated immunoassay platforms will have TT3 level below the 2.5th percentile measured by LC-MS/MS, and discrepancies are more frequent in the setting of elevated TSH [20,29,30]. It has been speculated that LC-MS/MS can perform better not only at low but at high thyroid concentrations [17], with significantly better log-linear relationship between fT4 and TSH in comparison to IA [11]. However, the comparison of LC-MS/MS and IA based on both central (TSH) and peripheral markers of thyroid hormone action has not previously been performed.

In this study, for the first time we have compared thyroid hormones measured by both IA and LC-MS/MS methods in euthyroid and hyperthyroid states in the same cohort of patients, and found no meaningful differences between methods. These data are consistent with an advantage of LC-MS/MS to measure analytes at low concentrations [26], but equal validity of IA and LC-MS/MS for detection of analytes at higher concentrations, as in our cohort of patients with hyperthyroidism.

Thus, based on our study, there is no difference in TH concentrations measured by IA *versus* LC-MS/MS in the mildly hyperthyroid state. The major strength of this study is the unique study design that included analysis of data performed on the same cohort of patients during euthyroid and hyperthyroid states, using the same samples for measurement of THs by IA and LC-MS/MS, thus eliminating inter-individual variability. Furthermore, thyroid tests were always measured at the same time time of day, thus minimizing effects of circadian rhythmicity of TSH and thyroid hormones on the results. Our major limitations are small sample size and use of a convenience sample of patients with mutations of the insulin receptor. These findings require confirmation in a larger sample of healthy subjects.

Acknowledgement

This work was supported by the Intramural Research Programs of the National Institute of Diabetes and Digestive and Kidney Diseases and the Clinical Center, National Institutes of Health, Bethesda , MD. The authors thank Douglas Joubert from NIH Library Writing Center for manuscript editing assistance and Robert Simple for performing the genetic testing.

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Citation: Kushchayeva Y, Soldin SJ, Stolze B, Yu X, Auh S, Lin TC, et al. Comparison of Thyroid Panel by Immunoassay and Liquid Chromatography-Tandem Mass Spectrometry during Transition from Euthyroid to Hyperthyroid State. Annals Thyroid Res. 2019; 5(1): 178-184.