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Thyroid hormone testing by tandem mass spectrometry

Offie P. Soldin^{a,b,c,d,*} and Steven J. Soldin^{b,d,e}

^aDepartment of Oncology, Georgetown University Medical Center, Washington, DC, USA

^bDepartment of Medicine, Georgetown University Medical Center, Washington, DC, USA

^cDepartment of Physiology and Biophysics, Georgetown University Medical Center, Washington, DC, USA

^dBioanalytical Core Laboratory, Georgetown University Medical Center, Washington, DC, USA

^eDepartment of Pharmacology, Georgetown University Medical Center, Washington, DC, USA

Abstract

In the euthyroid person, absolute free thyroxine concentrations remain constant and correlate with the tissue hormone level, its biologic effect and the metabolic status of the patient. However, most circulating thyroid hormone is bound to plasma proteins and only a minute amount is in the unbound free form. Studies have shown that current free thyroxine immunoassays are binding protein dependent. Novel high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) methods have successfully dealt with problems inherent in many immunoassays for thyroid hormones and afforded improved specificity and accuracy in thyroid hormone measurements.

We emphasize problems with thyroid hormone testing employing immunoassays including direct and indirect thyroid hormone immunoassays, sample processing, methods of free hormone separation and review the emerging role of liquid chromatography-tandem mass spectrometry in thyroid hormone testing. The latest generation of tandem mass spectrometers has superior limits of quantification, permitting omission of previously employed derivatization steps.

Liquid chromatography-tandem mass spectrometry affords the specificity, precision, and limits of quantification necessary for the reliable measurement of thyroid hormones, enhancing diagnostic capabilities, and affording the profiles of the iodothyronines and thyronamines. These methods are especially important in states of disease and during pregnancy when protein binding is a factor that interferes with other methods for thyroid hormone analysis.

Keywords

Thyroxine; Triiodothyronine; Free thyroxine; Iodothyronines; Thyronamines; Thyroid function tests; Immunoassays; Hypothyroid; Hypertyroid

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*Corresponding author. Lombardi Comprehensive Cancer Center, LL, S-166, Georgetown University Medical Center, 3800 Reservoir Road, N.W., Washington, DC 20057, USA. Fax: +1 202 784 3034. os35@georgetown.edu (O.P. Soldin).

Conflicts of interest statement

Upon manuscript submission, all authors declare no conflict of interest.

Author contributions

All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 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; and (c) final approval of the published article.

Background

The thyroid hormones, thyroxine (3, 3', 5, 5'-tetraiodo-L-thyronine, T₄) and triiodothyronine (3, 3', 5-triiodo-L-thyronine, T₃) are essential for cell differentiation, cellular metabolism, normal development and maintenance of normal neurological and physiological functions. Together with thyroid stimulating hormone (thyrotropin, TSH), circulating thyroid hormone concentrations indicate thyroid status and help in diagnosing thyroid disease status and treatment. Assays to determine thyroid hormone concentrations in serum or plasma include assays of total hormone and of free hormone concentrations. Immunoassay methods used in thyroid function testing in clinical settings lack specificity and are affected by numerous binding proteins and antibodies that limit their clinical utility [1].

Total thyroxine vs. free thyroxine

Thyroxine, synthesized solely by the thyroid gland, is deiodinated by tissue deiodinases to triiodothyronine—the active thyroid hormone. Circulating iodothyronines are bound by binding proteins including thyroxine binding protein (TBG), transthyretin (TTR, prealbumin) and albumin. Thyroxine is 99.96% bound to carrier proteins in the circulation, while 99.60% of triiodothyronine is bound [2]. Binding to carrier proteins renders thyroxine inactive, while the non-bound hormone, the free thyroxine is active and reflects the clinical availability of thyroxine. Moreover, binding protein concentrations are highly dependent on individual health status and are lower in disease [3]. Further, in some healthy situations, such as pregnancy, when serum albumin is normally decreased in concentrations, thyroxine binding proteins are elevated by as much as 50% [4]. The “free hormone” concept is generally accepted as an appropriate measure for thyroxine. This implies that the supply of thyroxine or of triiodothyronine into cells is governed by their unbound (free) concentrations in serum rather than the protein-bound fractions. Therefore, clinicians are mostly interested in obtaining serum-free thyroxine concentrations for the diagnosis of thyroid disease.

Free thyroxine and free triiodothyronine assays

Tests to measure the free fraction of thyroxine should reflect hormone concentration without bias despite large variations in serum binding protein concentrations and other factors that are known to affect immunoassays. The main purpose of free T₄ and free T₃ assays is to distinguish reliably between thyrotoxicosis, hypothyroidism, and the euthyroid state. This objective cannot be attained with assays of total T₄ and T₃ because of hereditary and acquired variations in the concentrations of binding proteins.

Current free T₄ immunoassay methodology has been specifically engineered to overcome common thyroxine binding globulin abnormalities. As a result, these tests include proprietary blockers and binders that make them subject to albumin sensitivity, decreasing diagnostic accuracy under conditions in which there are binding protein abnormalities—during pregnancy, nonthyroidal illness and congenital albumin abnormalities (familial dysalbuminemic hyperthyroxinemia).

Of the available methods for the estimation of the free hormone concentration in serum none are infallible in the evaluation of the thyroid hormone-dependent metabolic status. Immunoassays for the analysis of free T₄ include assays designed to analyze free T₄ concentrations following prior separation in the absence of protein bound T₄ such as the Nichols free T₄ equilibrium dialysis method, long regarded as the gold standard method of the 1990s and no longer commercially available. A different group of free T₄ assays are designed to analyze free T₄ concentrations in the presence of proteins (assays are applied to unfractionated samples of serum). These are commonly referred to as direct analogue FT₄ methods and essentially all of the commercially available immunoassays on

large chemistry platforms. This latter group has been strongly criticized for poor correlation with the former Nichols gold standard method or any of the recent equilibrium dialysis/ultrafiltration LC/MS/MS methods currently regarded as the new gold standard [5–10].

Direct analogue immunoassays, currently the most common method for the measurement of free thyroxine and free triiodothyronine, are available at most clinical laboratories and have historically been known for their inconsistent analytical performance and lack of correlation with log TSH [1, 5, 8, 11]. The disagreements reported between measurements performed on identical samples processed using different direct analogue immunoassay methods have led to questioning thyroid hormone immunoassay results and their clinical use [1, 5, 11]. All direct analogue immunoassays for free T4 use a “fudge factor” to estimate the free T4 in the sample and have been criticized over the years for poor quality. They are subject to interferences and lack of specificity. Among the factors known to interfere with direct analogue immunoassay measurements are issues originating in the sample, such as abnormal binding proteins, heterophilic antibodies, autoantibodies, intrinsic dilution and dialyzable protein binding competitors [12, 13]. In addition, factors such as assay antibodies, analogs and free fatty acids (FFAs) are also suggested to be a cause of interference in these bioassays [14–17].

Sample preparation by equilibrium dialysis and ultrafiltration prior to free T4 and free T3 sample analysis

Free thyroid hormone measurements require the physical separation of the free hormone fraction and the protein bound hormone prior to free hormone quantification. It is of particular importance that the separation step should not disturb the endogenous equilibrium [18, 19]. Currently the accepted gold standard for the measurement of free thyroxine is equilibrium dialysis (ED) or ultrafiltration (UF) of serum performed at 37 °C prior to hormone measurement in the dialysate or ultrafiltrate by either immunoassays or tandem mass spectrometry (MS/MS) [20]. However, equilibrium dialysis is labor intensive, imprecise, technically demanding, time consuming (17– 24 h), expensive and is not available in most clinical laboratories [21, 22]. The implicit dilution of small molecules is a limitation of equilibrium dialysis that needs to be overcome. Conversely, ultrafiltration is a promising alternative that is currently used in many North American Laboratories. It has the advantage of being less time consuming (30–40 min), it is easier to use and provides far superior reproducibility when compared to equilibrium dialysis although current methods can be accidentally leaky [20]. Other major concerns with both equilibrium dialysis and ultrafiltration include non-equilibrium issues, absorption, protein leakage, and temperature control [19].

Theoretically, both equilibrium dialysis and ultrafiltration can be valid methodologies to separate the free hormone prior to analysis. The primary difference between equilibrium dialysis and ultrafiltration is dilution. Studies have shown that the dilution used for equilibrium dialysis can mask the influence of inhibitors (i.e. drugs or free fatty acids) of thyroid hormone binding to proteins [23–25]. This could be a very significant issue causing low free T4 equilibrium dialysis values during pregnancy in which free fatty acids is high and the primary free fatty acids binder (albumin) is low. Studies show that in vitro normalization of albumin can normalize free T4 immunoassay values thus illustrating they do not measure the free hormone accurately [26, 27].

In addition to variability due to separation techniques, variability in the detection steps result in significant differences among results by different equilibrium dialysis-immunoassay or ultrafiltration-immunoassay methods [19].

Tandem mass spectrometry

The use of tandem mass spectrometry (MS/MS) following equilibrium dialysis or ultrafiltration has overcome the issues with the use of immunoassays in the measurement of thyroid hormones. Tandem mass spectrometry quantification of thyroid hormones in biological fluids is an alternative to the measurement of thyroid hormones using immunoassays. [6, 9, 10, 28–36]. The first T4 and T3 methods developed to measure total concentrations of based on tandem mass spectrometry relied on gas chromatography and isotope dilution mass spectrometry with selected ion monitoring required laborious sample cleanup and derivatization [6, 9, 28, 30, 36–39]. Further development was achieved by the use of high performance liquid chromatography coupled to mass spectrometry in selected ion monitoring mode, or MS/MS in multiple reaction monitoring (MRM) mode to measure total thyroxine and triiodothyronine [10, 28, 29].

Although the equilibrium dialysis LC/MS/MS procedure brings many potential benefits, it also poses some problems. In view of the aforementioned controversy regarding the validity of current immunoassay methods for measuring free thyroid hormone, interest in the development of in-house equilibrium dialysis or ultrafiltration LC/MS/MS procedures is great. Consequently, it is to be expected that many procedures will be developed worldwide, using different separation techniques (ultrafiltration or equilibrium dialysis) under different conditions (e.g., serum prediluted or not, pH buffered at 7.4 or not and performed at variable temperatures, resulting in non-uniformity).

Mass spectrometry measurements of hormones that bind to serum proteins will only be as good as the specimen preparation steps associated with their quantification. The majority of commercial and university hospital laboratories measuring free thyroid hormones employ either equilibrium dialysis or ultrafiltration at 37 °C.

Soldin et al. [9] were the first to develop a method to quantify free thyroxine using ultrafiltration followed by liquid chromatography tandem mass spectrometry. This method employs ultrafiltration, followed by liquid chromatography with deuterated internal standards and a C-18 3 cm Supelco column and utilized MRM for quantification in the negative mode. The method was further improved when the Applied Biosystems API-5000 became available allowing the development of a simultaneous measurement of free T4 and free T3 due to the enhanced sensitivity of the API-5000 [6]. The most recent version uses a different column, a gradient and ultrafiltration performed at 37 °C. Tandem mass spectrometric methods employing either ultrafiltration or equilibrium dialysis and which permit the simultaneous measurement of free T4 and free T3 have been published by groups working at Georgetown University and ARUP laboratories [6, 9, 31, 40]. A procedure based on equilibrium dialysis combined with determination of thyroxine in the dialysate with a trueness-based measurement procedure has been proposed but not validated as a candidate international conventional reference measurement [38, 41].

The method of van Uytendange et al. [42] reported the quantification of free thyroxine using equilibrium dialysis-liquid chromatography-tandem mass spectrometry. The dialysate, with $^{13}\text{C}_6\text{-T}_4$ as internal standard and $^{13}\text{C}_9\text{-T}_4$ as carrier, was purified using solid-phase extraction. A 15-cm C-18 column provided 16-min liquid chromatography separation before detection in positive MRM mode. Using these methods on-line sample preparation techniques were employed, and high-end liquid chromatography-tandem mass spectrometry instrumentation with sufficient sensitivity for measurement of thyroid hormone concentrations at the pg per mL level were required.

Ultrafiltration or equilibrium dialysis tandem mass spectrometry affords a superior detection alternative to thyroid hormone quantification using equilibrium dialysis-immunoassays by

providing a faster technique with higher specificity. Tandem mass spectrometry provides specificity because of its ability to monitor selected mass ions and sensitivity because of the enhanced signal-to-noise ratio. Such analysis of serum free thyroid hormones has helped to resolve some of the controversies regarding the validity of free thyroid hormone determinations by immunoassays.

Liquid chromatography-tandem mass spectrometry is an excellent tool for documenting the known physiological phenomenon of a log-linear relationship between TSH and thyroid hormone concentrations [43]. We conducted a prospective study to determine the performance of liquid chromatography-tandem mass spectrometry in documenting both group and individual relationships between thyroid hormone and TSH concentrations [43]. Thyroxine, triiodothyronine, free T4 and TSH levels of 50 euthyroid patients undergoing thyroidectomy were documented on two occasions prior to thyroidectomy and following thyroidectomy once patients achieved either a normal or low serum TSH concentration. Thyroid hormone concentrations were documented by both immunoassay and liquid chromatography-tandem mass spectrometry, and their relationship with TSH was studied both in the entire group and in individual patients pre- and post-thyroidectomy. Free T4 correlated better with the log-transformed TSH when measured by liquid chromatography-tandem mass spectrometry. Post-thyroidectomy the closest correlation was between log TSH and free T4 ($r = 0.86$, $p < 0.001$) [7]. In contrast, immunoassay for free T4 showed a poor correlation with log TSH [8]. Most importantly, the ultrafiltration tandem mass spectrometry methods also correlate well with log TSH in both the pediatric and adult populations while this is not the case for direct analogue immunoassays [7, 8].

However, tandem mass spectrometry of thyroid hormones can be subject to interference, especially through ion suppression effects. Ion suppression results from the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector. Materials shown to cause ion suppression, include salts, ion-pairing agents, endogenous compounds, drugs, metabolites, and proteins [44].

Whenever mass spectrometric assays are developed, ion suppression studies should be performed using expected physiologic concentrations of the analyte under investigation. Ion suppression can be minimized by enhancing specimen cleanup prior to analysis, utilizing gradients that help separate interferences from the analyte of interest, and assess internal standard peak heights (signal) which should remain more or less constant between samples. We found that employing such appropriate gradients can eliminate or greatly reduce ionization inhibition effects.

Factors meriting consideration for thyroid hormones using tandem mass spectrometry

Derivatization vs. nonderivatization

This is currently an important issue, with advocates on both sides. Derivatization proponents claim that both enhanced limit of quantification and specificity can be achieved by adopting this approach. This is however, questionable since derivatization has its disadvantages, which include decreased precision due to the added derivatization steps (extraction, derivatization at an extreme of pH). Derivatization methods are also lengthy and time-consuming. The detection limits with modern mass spectrometers have improved greatly since inception and have made the derivatization of analytes unnecessary. For this reason, we have been able to avoid derivatization approaches for the measurement of thyroid hormones [6, 9, 10].

Type of ionization

Electrospray ionization (ESI) in the positive mode yields the best results for the iodothyronines (thyroxine, triiodothyronine, diiodothyronine and the thyronamines). The method used, which avoids derivatization, has a lower limit of detection (LLOD) of 2.5 pg/mL for all iodothyronine analytes when run on the API-5000 tandem mass spectrometer (Applied Biosystems). Total sample requirement is only 0.2 mL and total chromatography using an Agilent Zorbax SB C-18 (2.1 × 30 mm, 1.8 micron column) [39]. For thyroid hormones, atmospheric pressure photoionization (APPI) has no advantages over ESI [39]. Although ion suppression has limited the usefulness of many tandem mass spectrometry methods, it is a rare occurrence with the method described. Moreover, the use of deuterated internal standards and an online sample wash step are partly responsible for the good performance.

Lack of standardization of thyroid hormone assays

Of issue is the lack of standardization of thyroid hormone assays and it is a major deficiency in epidemiologic studies. Moreover, traditional proficiency testing materials do not adequately reflect thyroid function testing biases [45]. Inproficiency testing surveys, there are differences in the values reported by users of various analytic methods. Two contributors to this variation are calibrator bias and matrix effects of proficiency testing materials. The College of American Pathologists (CAP) Chemistry and Ligand surveys enrolled approximately 3900 clinical laboratories and quantified the biases of the analytic methods used to measure thyroxine, triiodothyronine, free thyroxine, and free triiodothyronine levels. The means and SDs for each analytic method were determined and for thyroxine and triiodothyronine also by isotope dilution liquid chromatography tandem mass spectrometry. Other target values in the study were the median of the means obtained for the various analytic methods. Calibration biases were evaluated against limits based on intra- and inter individual biological variation. 11 of the 13 methods for free thyroxine failed to meet the goals for bias. The failure rates were 12 of 16 methods for thyroxine, 8 of 11 for triiodothyronine, and 9 of 11 for free triiodothyronine. Further studies indicate the normal reference intervals for different sexes, age, and ethnicities are variable, with normal values differing by a factor of approximately 3 [45]. This supports the CAP proficiency testing data reported above.

Tandem mass spectrometry and thyroid function tests in pregnancy

Thyroid disease is known to have a number of deleterious effects on pregnancy, the developing fetus, and during the postpartum period on the mother and newborn. Complications of hypothyroidism include miscarriage, preterm delivery, decreased intelligence quotient, visual-motor deficiencies in the children born to hypothyroid women and postpartum thyroiditis [46–48]. Thyroid disease diagnosis and monitoring are complicated during pregnancy and lactation, due to pregnancy-related changes in thyroid hormone metabolism [49]. Although TSH (and not free T4) is the primary test of thyroid status during pregnancy, just as with non-pregnant subjects it is important to obtain concise free T4 measurements and especially during pregnancy. Therefore, the diagnosis and monitoring of thyroid disease necessitates awareness to the technical limitations of current thyroid-related biochemical tests.

Dramatic changes occur in thyroxine and triiodothyronine concentrations during pregnancy and pose challenges in the management of thyroid disease in pregnancy. Very early in pregnancy, levothyroxine replacement needs to be increased [50, 51]. For reasons that are still not entirely clear, although dependent in part on changes in thyroxine binding, free thyroxine levels decrease as pregnancy progresses necessitating the use of trimester-specific

reference intervals for appropriate replacement [52, 53]. Thyroxine binding protein levels vary by hormonal status, iodine sufficiency, ethnicity and disease states and are higher in pregnancy, hence the importance of free T4 assays to measure the unbound hormone. The need to reliably identify hypothyroxinemic pregnant patients, especially in the first trimester, is of prime importance for early fetal brain development before the fetal thyroid functions [47]. Therefore free T4 tandem mass spectrometry is of importance especially during pregnancy, when protein levels are abnormal.

Tandem mass spectrometry has been particularly useful for the determination of trimester-specific reference intervals for the iodothyronines and for the study of levothyroxine pharmacokinetics in pregnant and nonpregnant women [54–57]. Trimester-specific free T4 reference intervals are clinically significant for the anti-thyroid drug treatment of Graves' hyperthyroidism and in for pregnancy patients who are hypothyroxinemic without having abnormal TSH.

Using equilibrium dialysis and LC/MS/MS, the 95% FT4 reference intervals decreased gradually with advancing gestational age: from 1.08–1.82 ng/dL in week 14 to 0.86–1.53 ng/dL in week 20 [31]. Using ultrafiltration followed by isotope dilution LC/MS/MS, serum FT4 concentrations (given as mean $\pm$ SE) were 0.93 ± 0.25 ng/dL in nonpregnant women, 1.13 ± 0.23 ng/dL in the 1st trimester, 0.92 ± 0.30 ng/dL in the 2nd trimester, and 0.86 ± 0.21 ng/dL in the 3rd trimester [52]. FT4 measured in the same samples by a direct analog immunoassay the values were: 1.05 ± 0.22 ng/dL, 0.88 ± 0.17 ng/dL and 0.89 ± 0.17 ng/dL in the 1st, 2nd and 3rd trimester respectively. Serum FT4 by LC/MS/MS correlated very well with serum FT4 measured by classical equilibrium dialysis, but correlation with results from the FT4 immunoassay were less satisfactory [52]. Free thyroid hormone concentrations measured by LC/MS/MS correlate to a greater degree with log TSH values compared to concentrations measured by immunoassay [7].

The application of LC/MS/MS for measurement of free thyroid hormones is currently in routine clinical use in a few centers. Assays can be rapidly performed and a large volume of samples can be processed. The high equipment costs can be paid off with high throughput in a reasonable time. The method is ideally suited for generating reliable, reproducible trimester-specific reference ranges for FT4 [52]. A working group of the International Federation of Clinical Chemistry and Laboratory Medicine recommends the use of isotope dilution-LC/MS/MS for measuring T4 in the dialysate from equilibrium dialysis of serum in order to obtain a trueness-based reference measurement procedure for serum FT4 [58].

Future directions

Tandem mass spectrometry now affords the specificity, precision and limits of quantification necessary for the reliable measurement of thyroid hormones in human fluids, thereby enhancing diagnostic capabilities, particularly when thyroid hormone profiles are available. LC/MS/MS has great potential to be applied in the routine clinical assessment of free T4 and free T3 in the near future. Tandem mass spectrometry now provides profiles of the iodothyronines and thyronamines [59, 60]; 3-Iodothyronamine (3-T1AM) and thyronamine (TAM) are endogenous signaling molecules that exhibit great structural similarity to thyroid hormones but apparently antagonize classical triiodothyronine actions [61].

Major advantages of tandem mass spectrometry include small sample size, the simultaneous measurement of many analytes, and enhanced specificity compared to immunoassay methods. Mass spectrometric methods are still fairly labor intensive, and certainly require a higher level of laboratory expertise than do immunoassay platforms. The value of hormone profiles will likely be more appreciated in the years ahead. Although mass spectrometric

assays are not always more precise than immunoassays, they are more specific for measuring the analyte of interest. By omitting extraction and derivatization steps, tandem mass spectrometric procedures have good reproducibility providing low CVs for both within and between day studies [6, 9]. Provided gradient elution is used these methods are also relatively free of ion suppression.

Conclusions

Advanced mass spectrometry for quantifying thyroid hormones in biological fluids provides better quantification because of greater analytical specificity and less analytical interference than immunoassays. Such techniques have been applied to total thyroxine [10, 28, 29, 62] and free thyroxine determinations [6, 9, 31, 40, 58]. Tandem mass spectrometry measurements of thyroid hormones will only be as good as the specimen preparation steps associated with their quantification. Total hormone assays will require complete hormone release from protein–hormone complexes, while free thyroid hormone assays require a physical separation of free thyroid hormone and protein bound hormone prior to quantification. To accomplish these goals, new separation technologies may be needed before any method can be regarded as a gold standard. While the implicit dilution of small molecules is a limitation of equilibrium dialysis that needs to be overcome the current methods of ultrafiltration are more amenable to routine quantitation in the clinical laboratory. Ideally, the free hormone reference method would be a technique that employs ultrafiltration at 37 °C to avoid dilution effects and the direct measurement of free hormone in the ultrafiltrate by mass spectrometry.

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