CHAPTER FOUR

The Expanding Role of Tandem Mass Spectrometry in Optimizing Diagnosis and Treatment of Thyroid Disease

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Advances in Clinical Chemistry, Volume 61
ISSN 0065-2423
http://dx.doi.org/10.1016/B978-0-12-407680-8.00005-1

Abstract

This review discusses the state-of-the-art measurement of free and total thyroid hormones in clinical laboratories. We highlight some of the limitations of currently used immunoassays and critically discuss physical separation methods for the measurement of free thyroid hormone. Physical separation methods, such as equilibrium dialysis or ultrafiltration, followed by tandem mass spectrometry for the measurement of free thyroid hormones offer many advantages, which we feel, can deepen our understanding of thyroid hormone metabolism and improve patient diagnosis and care. Problems with direct analogue immunoassay methods for FT_4/FT_3 as well as immunoassay methods for total T_3 at low T_3 concentrations and during pregnancy are highlighted. Improved diagnosis and patient management can be achieved utilizing tandem mass spectrometry for these measurements.

1. INTRODUCTION

Overview: Thyroid hormones play an integral role in growth, energy homeostasis, maintenance of physiological function and are essential for normal development. The accurate assessment of thyroid function is therefore important for both the diagnosis and management of thyroid disease. Thyroid function is routinely assessed by the measurement of thyroid-stimulating hormone (TSH) and thyroid hormones; thyroxine (T_4), triiodothyronine (T_3), free thyroxine (FT_4), and free triiodothyronine (FT_3).

Although T_4 is the main secretory product of the thyroid gland, T_3 is generally considered the biologically active hormone. Most of the T_3 is derived from peripheral deiodination of T_4, and three monodeiodinase isoenzymes have been identified in the deiodination of T_4 [1,2]. Type I and Type II 5′-deiodinase generate the active hormone; 3,3′,5-triiodothyronine by reductive deiodination of the phenolic ring of T_4. Type I deiodinase expression is upregulated by T_3 and expressed in liver, kidney, thyroid, and pituitary. Type II deiodinase is localized in the endoplasmic reticulum and downregulated by T_3. Type III deiodinase inactivates T_4 and T_3 by deiodination of iodothyronines at the tyrosyl ring yielding the biologically inactive reverse T_3 (rT_3) and reverse diiodothyronine (rT_2) [1–4].

In serum, the majority of T_4 and T_3 circulates bound to high concentration low-affinity proteins, mostly albumin and transthyretin, and to a low concentration high-affinity binding protein, namely thyroxine-binding globulin (TBG) [5]. Binding to these proteins increases their biological half-life and enables their transport. Only a small percentage of total

thyroxine (TT_4) and total triiodothyronine (TT_3) circulates as free hormone. Based on the free hormone hypothesis, it is widely accepted that the free fraction is biologically active and, therefore, of most interest to monitor in patients with thyroid disorders [5–8]. Accurate and precise measurements of TT_4, TT_3, FT_4, and FT_3 are important for the diagnosis, treatment, and ongoing monitoring of patients with thyroid disease.

Assays for the measurement of free thyroid hormone can be broadly divided into two categories: those that employ a physical separation step, such as ultrafiltration or equilibrium dialysis, to separate the free fractions from binding proteins before measurement or those that estimate FT_4 and FT_3 without a physical separation step. The first assays for the measurement of FT_4 and FT_3 used equilibrium dialysis to separate serum proteins from FT_4 and FT_3 prior to the measurement by radioimmunoassay [9,10]. However, the measurement of free thyroid hormone by equilibrium dialysis is labor intensive and time consuming and, in practice, most clinical laboratories currently use direct (analogue) immunoassays for the measurement of FT_4 and FT_3 [11–13], which rely on the measurement of FT_4 and FT_3 in diluted serum without prior separation of the binding proteins. The validity of free thyroid hormone measurement by direct analogue immunoassay has many limitations, is controversial, and is still debated [11,14–29].

This review discusses the state-of-the-art measurement of free and total thyroid hormones in clinical laboratories. We highlight some of the limitations and clinical conditions where these methods may be inaccurate and critically discuss physical separation methods for the measurement of free thyroid hormone.

1.1. The inverse log–linear relationship between FT_4 and TSH

Because of the critical role of thyroid hormones, their concentration is tightly regulated; this is mainly achieved by a negative thyroid pituitary hypothalamic feedback loop. TSH secretion is upregulated in case of decreased free thyroid hormone levels and suppressed in response to increased hormone concentrations.

The relationship between FT_4 and TSH is often described as an inverse log–linear relationship [12,30,31]. An understanding of the inverse log–linear relationship between FT_4 and TSH is critical when interpreting thyroid function results and should in addition provide an important tool to evaluate assays.

FT_4 measured by ultrafiltration or equilibrium dialysis followed by liquid chromatography tandem mass spectrometry showed (LC–MS/MS) a far

Table 4.1 Correlation of thyroid hormone levels with log-transformed TSH

Platform	Correlation coefficient (*R*)	References
Immunoassay studies		
Siemens R × L dimension	0.58 (post-thyroidectomy)	[27]
	0.08 (pre-thyroidectomy)	[27]
	0.48 (FT_3)	[32]
Abbott Architect Ci8200	0.05 (FT_4 in females)	[26]
	0.01 (FT_4 in males)	[26]
Siemens Immulite 2500	0.45 (FT_4)	[24]
Beckman Coulter Access DXI 800 Unicel	0.75 (FT_4)	[29]
Roche Modular E170	0.76 (FT_4)	[29]
Siemens ADVIA Centaur	0.72 (FT_4)	[29]
Mass spectrometry studies		
AB Sciex 5000	0.90 (FT_4)	[32]
AB Sciex 5000	0.77 (FT_4)	[32]
AB Sciex 5000	0.84 (FT_4)	[24]
AB Sciex 5000	0.86 (FT_4)	[27]

better correlation with log-transformed TSH compared to immunoassays [24,26,27,29,32] (see Table 4.1; Fig. 4.1). Serdar *et al.* evaluated the relationship between FT_4 and log TSH on three different immunoassay platforms and found a relatively poor correlation on all three [29]. The reason that the studies performed by Serdar *et al.* [29] resulted in a better (but still suboptimal) correlation between immunoassay FT_4 and log TSH than those performed by other investigators [24,26,27,29,32] is that Serdar's studies were performed on a largely euthyroid population with very few hypothyroid individuals included. Even with this slanted patient population, their correlations of FT_4 with log TSH were not as good as those achieved utilizing ultrafiltration tandem mass spectrometry [24,26,27,29,32].

Recently, Clark *et al.*, measuring TSH and FT_4 by an immunoassay, suggested that the relationship between FT_4 and log TSH may be better described by a fourth order polynomial equation [28]. Hoermann *et al.* further defined the relationship between FT_4 and TSH and proposed a

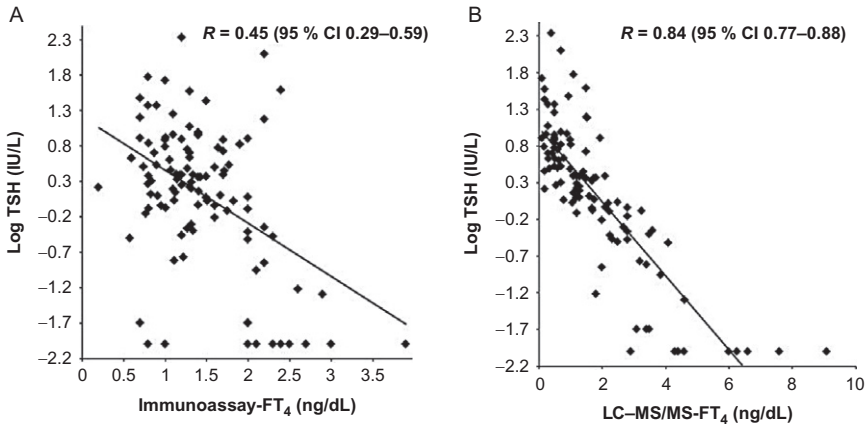

Figure 4.1 Inverse log–linear relationship between log TSH and (A) immunoassay-FT_4 and (B) LC–MS/MS-FT_4. The inverse log–linear Pearson correlation between log TSH and LC–MS/MS-FT_4, 0.84 (95% CI 0.77–0.88), was significantly better ($P < 0.0001$) than between log TSH and immunoassay FT_4, 0.45 (95% CI 0.29–0.59) [24]. *Data from [24].*

nonlinear error function in which there is an increasingly stronger TSH response depending on the extent of deviation of FT_4 from a putative optimum set point [33]. In other words, the slope of the inverse log–linear relationship between TSH and FT_4 varies with the distance from a mean FT_4 set point. Evaluation of these equations that further refine the relationship between TSH and FT_4 as well as potential factors affecting the relationship between FT_4 and TSH will benefit from being further examined with gold standard FT_4 methods less prone to analytical interference. It is also important to keep in mind that every individual has his/her own intraindividual set point for FT_4 and TSH determined by genetic and environmental factors [34].

Some conditions where the inverse log–linear relationship between FT_4 and TSH is disturbed include hypothalamic and pituitary failure, resistance to thyroid hormones or TSH, TSH-secreting tumors, nonthyroidal illness, drugs that may cause suppression of TSH secretion such as dopamine and glucocorticoids, patients on thyroxine replacement therapy that has not reached stead state [35], and patients with macro-TSH [36].

1.2. Relationship between T_3 and TSH

A poor correlation for T_3 between immunoassay and LC–MS/MS has been documented [37]. In addition, Jonklaas *et al.* demonstrated a lower median and mean T_3 when measured by LC–MS/MS compared to when measured

by immunoassay in a group of individuals with TSH more than 4.5 mIU/L and a higher median and mean T_3 when the TSH's were less than 0.35 mIU/L [38]. These studies demonstrate that the inverse relationship between T_3 and TSH is also better when measured by LC–MS/MS than when measured by immunoassay.

2. MEASUREMENT

2.1. Total thyroid hormone measurement

Total hormone assays necessitate the inclusion of a displacing agent such as 8-anilino-1-napthalene-sulfonic acid or salicylate to release the hormone from binding proteins before measurement of T_4 or T_3 by a competitive immunoassay [12]. TT_4 and TT_3 measurement by LC–MS/MS has also been described [37,39]. These methods use a protein precipitation step followed by online extraction before introduction of the sample into the mass spectrometer.

2.2. Immunoassays: Free thyroid hormone measurement

Most clinical laboratories use direct (analogue) immunoassays for the measurement of FT_4 and FT_3 [11,13,22]. These methods can be divided into two categories: one-step assays that are designed to give a signal inversely proportional to the free hormone concentration in the presence of binding proteins and two-step assays that separate a fraction of the FT_4 and FT_3 pool from the binding proteins before the assay is performed [11].

2.2.1 One-step, labeled hormone analogue methods

The one-step assays are dependent on the use of hormone analogues that are chemically modified to have a molecular structure that, in theory, prohibits it from interacting with binding proteins but retain the ability to compete with thyroid hormone for unoccupied hormone antibody sites. The hormone analogue, a signal molecule labeled with an isotope or enzyme, competes with free hormone for a limited number of antibody-binding sites in a classical competitive immunoassay format in which the signal is usually inversely proportional to the free hormone concentration. The signal output is then converted to a free hormone concentration using calibrators with free hormone values assigned by a method employing physical separation.

Two main formats used in one-step assays are either a solid-phase antibody with labeled hormone analogue or a solid-phase hormone analogue

with labeled antibody with the labeled antibody approach taking preference in more recent assays [40].

Analogues are small-molecular-weight compounds originally developed to not bind to TBG; however, analogues still bind to albumin to varying degrees [18,22,41]. Although the one-step assays were considered to have good performance in conditions of increased TBG concentration, it has been shown that they tend to have poor diagnostic accuracy in the presence of abnormal albumin concentrations.

2.2.2 Two-step, labeled hormone assays

During a first incubation step, the two-step assays use a high-affinity anti-hormone antibody bound to a solid support to sequester a very small proportion of total hormone from a diluted serum specimen. These assays are designed to sequester only a very small proportion of total hormone in order to cause a minimal disturbance in the original serum bound-free equilibrium. After a short incubation period, the immobilized antibody containing bound T_4 or T_3 are washed to remove all unbound constituents before the second step is performed in which sufficient labeled hormone is added to bind to all the unoccupied antibody-binding sites. The amount of labeled hormone bound to the solid-phase antibody is quantified relative to gravimetric standards or calibrators that have free hormone values assigned by a reference method. The key feature of the two-step assay is that labeled hormone is physically prevented from interacting with binding proteins and is therefore, at least in theory, independent of the influence of serum proteins. A potential danger of these immunoassays is that the free hormone equilibrium may be disturbed when free hormone is sequestered.

2.3. Conditions in which free thyroid hormone measurement is impaired

The validity of free thyroid hormone measurement by direct analogue immunoassay is still debated [14,15,17,22] and has many limitations. The immunoassays rely on the assumption that the sample and standard are identical in all measured characteristics other than the concentration of analyte being measured [11].

2.3.1 Changes in binding proteins

Immunoassays for FT_4 become unreliable when the plasma protein binding is different between standard and sample as happen in changes in binding protein concentration or binding protein competitors.

FT_4/FT_3 methods should be able to accurately reflect the free hormone concentration without any contribution from the bound fraction. There is, however, some evidence that protein-bound T_4 can contribute substantially to analogue-based FT_4 estimates [13]. Fritz *et al.* demonstrated that analogue-based FT_4 immunoassays correlated closely with TT_4 concentration but failed to accurately detect huge variations in dialyzable FT_4 concentrations [16].

It is important that any assay used for the measurement of FT_4 is valid for the whole range of serum binding protein concentrations likely to be encountered in clinical practice [42]. A critical limitation of immunoassays is that these methods are dependent on protein binding concentration [13,20,21,43–47].

Understanding the factors that influence binding of T_4 and T_3 to these proteins and factors that influence the concentration of these binding proteins are critical both for developing and for evaluating FT_4/FT_3 assays. Common conditions that are known to cause changes in binding protein concentration, thereby affecting the accuracy of free hormone measurement include pregnancy, renal failure, and nonthyroidal illness.

2.3.2 Pregnancy

It is estimated that hypothyroidism may occur in up to 2.5% of pregnant women [48,49]. The fetal thyroid gland only begins concentrating iodine and synthesizing thyroid hormones after 12 weeks of gestation. Any requirement for thyroid hormones before this time is solely supplied by the mother [50,51]. It is becoming increasingly clear that maternal hypothyroidism is associated with reduced neuropsychological development as well as maternal obstetric complications [52–57]. Subclinical maternal hypothyroidism may also be associated with poor pregnancy outcomes such as placental abruption, preterm birth, and low-birth weight infants [58,59]. The detection, appropriate management, and monitoring of these cases are therefore critical to prevent adverse maternal and fetal outcome.

Pregnancy poses unique challenges to FT_4/FT_3methodologies and serum FT_4/FT_3 testing in pregnancy is known to be challenging for most of the current routinely available immunoassay methods [60].

The pregnancy associated increase in estrogen leads to increase glycosylation of TBG that retards the clearance of TBG leading to an increase in TBG concentration plateauing at about two to three times prepregnancy levels by 20 weeks of gestation [61,62]. The Law of Mass Action dictates

that some lowering of FT_4 may be expected in pregnancy because of the high TBG state.

It would be expected that this change would be accompanied by an increase in TSH secretion to restore the serum FT_4 concentration toward normal. Paradoxically, the increases in serum TBG and TT_4 in the first trimester coincide with subnormal levels of serum TSH, a phenomenon attributed to the thyroid-stimulating activity of human chorionic gonadotropin (hCG) which has structural homology with pituitary TSH [63,64]. The peak in hCG and the nadir of serum TSH occur together at about 10 weeks gestation [62,65,66].

In the second and third trimesters, both measured FT_4 and FT_3 decrease to approximately 20–40% below the normal mean [62,65,66]. The amount of decrease is method dependent and should be an important consideration when guidelines are established for the management of hypothyroidism in pregnancy.

In pregnancy, there is also a progressive decline in albumin concentration. Some of the method dependent decrease in FT_4 concentration may be due to the albumin dependence of specifically the one-step assays [67]. If serum albumin is subnormal, FT_4 estimates by analogue tracer methods tend to be low because more tracer is available in the sample than in the standard [20].

Some controversy exists regarding whether the observed decrease in FT_4 concentration is an analytical artifact or a true physiological phenomenon [68]. A decrease in FT_4 in the third trimester of pregnancy has been demonstrated with both immunoassays, equilibrium dialysis [69] and ultrafiltration [23] tandem mass spectrometry methods, making it likely that at least some of the decrease in FT_4 concentration is real [68].

It is imperative for clinicians taking care of pregnant patients to have access to accurate and reliable assays for FT_4 measurement. The American Thyroid Association currently recommends that the optimal method for the measurement of FT_4 in pregnancy is measurement of T_4 in dialysate or ultrafiltrate followed by LC–MS/MS [70].

Disagreement between methods remains a perplexing problem for clinicians involved in the follow up of these patients. Clinicians should be aware of gestational age-specific reference intervals. Unfortunately, poor comparability between various immunoassays available on the market poses a huge challenge. The American Thyroid Association guidelines for the diagnosis and management of thyroid disease during pregnancy and postpartum recognizes the limitations of current immunoassays for the measurement of FT_4

and recommends that in view of the wide variation in FT_4 results by immunoassays method-specific and trimester-specific reference ranges of FT_4 be applied [70]. Both clinicians and laboratory directors often remain unaware of changes if FT_4 reference intervals during pregnancy and few laboratories quote method and trimester-specific reference intervals for the population that the laboratory services. As current FT_4 and FT_3 immunoassay methods are unreliable in pregnancy due to many factors including the presence of nonspecific heterophilic antibodies our recommendation is to separate all thyroid hormone binding proteins by either ultrafiltration or equilibrium dialysis followed by either immunoassay or mass spectrometric quantitation. FT_4 index or TT_4 measurements can also be considered as potential alternatives [60]. In all instances, the use of pregnancy-specific reference ranges is important.

The accurate and precise measurement of T_3 in pregnancy may be of importance both clinically and for research. The presence of Type III deiodinase in the placenta indicates a role in modulating the thyroid status of the human fetus [71]. TT_3 in pregnancy measured by immunoassay correlated poorly with a LC–MS/MS method [72]. It has also been shown that the TT_3 measurement by immunoassay underestimates TT_3 measured by LC–MS/MS in each trimester as well as postpartum [73]. Further studies evaluating the analytical and clinical implications of TT_3 and FT_3 in pregnancy are warranted.

2.3.3 Renal failure

Chronic kidney disease affects thyroid function in many ways which include low concentration of circulating thyroid hormones, altered peripheral metabolism of thyroid hormones, and decreased binding of thyroid hormones to binding proteins [74]. It has been shown that in renal failure analogue assays can underestimate FT_4 values by as much as 40% in predialysis samples [75]. It is thought that some of this decrease may be explained by retained organic acids that can displace the tracer from albumin making more tracers available in the sample than in the standard leading to apparent lower serum FT_4 values [17,75]. Low serum FT_4 in hemodialysis patients and in patients with nephrotic syndrome may also be, as in pregnancy, due to a low albumin concentration [76].

2.3.4 Nonthyroidal illness

Critical illness can cause profound changes in thyroid hormone metabolism, these changes are often referred to as “euthyroid sick syndrome” or

"nonthyroidal illness." It is estimated that nonthyroidal illness may affect up to 70% of hospitalized patients. Nonthyroidal illness syndrome is typically associated with low T_3, possibly increased rT_3, low T_4, and increased TSH [77].

In acute events, such as sepsis or coronary bypass surgery, circulating levels of the binding proteins are low and nonthyroidal illness is generally associated with a decrease in FT_4 concentration. Some of this decrease may be assay related. Csako *et al*. showed that in nonthyroidal illness, low albumin concentrations are often accompanied by falsely low FT_4 concentration [41].

Equilibrium dialysis and ultrafiltration assays provide FT_4 estimates in the normal range in nonthyroidal illness with low TT_4 values and normal TSH's and are able to differentiate euthyroid patients from hypothyroid patients with comparable TT_4 levels. This distinction is important for clinicians taking care of acutely ill patients, some of whom may benefit from thyroxine replacement therapy. In contrast to the physical separation methods, analogue immunoassays often give falsely low FT_4 measurements in the low TT_4 (normal TSH) nonthyroidal illness patients and are often unable to distinguish these patients from hypothyroid patients [78].

The effect of medication on thyroxine concentration *in vivo* needs special consideration and will be discussed separately. Similar to drugs that may displace thyroxine from binding proteins, it has been postulated that disease-specific circulating endogenous compounds may also displace T_4 from its low-affinity protein binding sites on albumin [79–81].

The accurate and precise measurement of FT_4, FT_3, and rFT_3 using ultrafiltration tandem mass spectrometry is likely to further increase our understanding of nonthyroidal illness and possibly improve our management of these patients.

2.3.5 Drugs

Numerous drugs can displace T_4 and T_3 from its binding proteins and may therefore have both *in vivo* and *in vitro* effects on thyroid tests. Some drugs such as salicylate, phenytoin, carbamazepine, or furosemide may inhibit thyroid hormone binding to serum proteins in the specimen, displacing T_4 and T_3 from their binding proteins leading to an acute increase in the availability of FT_4 or FT_3 [82,83]. After displacement of FT_4 from binding proteins, a new equilibrium can be established *in vivo*. The withdrawal of drug at this point would cause an initial fall in FT_4 as more carrier protein becomes available, with renormalization of FT_4 as the equilibrium is reestablished through an increased release of hormone from the thyroid gland. The time scale and

magnitude of these competitor effects differ with the half-life of the competitor agent.

Any methods that employ a dilution step will result in a decrease in the concentration of the competitor drug, leading to more FT_4 binding to binding proteins *in vitro* and to a falsely low estimation of FT_4. Current FT_4 assays that employ a dilution factor may therefore fail to accurately measure FT_4 in the presence of binding protein inhibitors and the hormone displacement effect of the drugs may be underestimated upon dilution.

Intravenous heparin administration, through *in vitro* stimulation of lipoprotein lipase liberates free fatty acids (FFAs) from triglycerides, the FFAs inhibit T_4 binding to serum proteins and thereby spuriously increase the FT_4 measured.

The effect of drugs on protein binding concentration also needs consideration. Estrogen, tamoxifen, heroin, methadone, and 5-fluoracil may all cause an increase in TBG concentration [11,61,84]. TBG may be decreased corticosteroids and androgens.

2.3.6 Genetic abnormalities in binding proteins

Familial dysalbuminemic hyperthyroxinemia is often cited as a reason for spuriously elevated FT_4 measurement [85,86]. In these patients, an Arg-His substitution at position 218 [87] leads to an increased affinity of albumin for T_4 and T_4 analogues, resulting in a spuriously high estimation of FT_4 when direct analogue immunoassays are used to estimate FT_4. Congenital TBG excess and deficiency may also result in inaccuracies in the measurement of FT_4 [11].

2.3.7 Heterophile and autoantibodies

Immunoassays are susceptible to heterophile antibody interference. Heterophile antibodies present in most of the pregnant women may cause falsely low or falsely high values of thyroxine, depending on the nature of the interfering antibody or the assay design [88,89]. Autoantibodies directed against T_4 or T_3 are another source of potential misleading results [89–91]. The presence of rheumatoid factor may also cause misleading results when immunoassays are used to measure FT_4 [92].

3. PHYSICAL SEPARATION METHODS

The gold standard separation methods for the measurement of free hormone are considered to be equilibrium dialysis or ultrafiltration, in which free hormone is first separated from that bound to binding proteins, followed

by measurement of the free hormone by a highly sensitive and specific assay [22,93–95].

The separation step used requires careful consideration [93]. It is important that the balance between the bound and free fraction of the analyte is not altered [42,93] as the accurate measurement of FT_4 depends on nondisturbance of the free hormone equilibrium [93].

3.1. Equilibrium dialysis

Equilibrium dialysis methods are considered to be among the best methods for the measurement of free thyroxine. The basic principle behind equilibrium dialysis is that two solutions are separated by a semipermeable membrane, allowing sufficient time to pass, the concentration of diffusible substances will be equal on both sides of the membrane. In the case of FT_4 measurement by equilibrium dialysis, the semipermeable membrane separates serum from the dialysis solution. Proteins and T_4 bound to proteins are unable to diffuse to the other side of the membrane due to size. FT_4 diffuse across the semipermeable membrane until equilibrium is reached.

The Nichols FT_4 equilibrium dialysis radioimmunoassay method (Nichols Institute, San Juan Capistrano, CA) was long regarded the gold standard method for the measurement of FT_4, but the kits are no longer commercially available. More recently, equilibrium dialysis methods for the measurement of FT_4 using a dialysis plate with 5 kDa molecular weight cutoff cellulose membranes were described by Van Uytfanghe *et al.* [96] and Yue *et al.* [97].

Various factors need to be considered when evaluating an equilibrium dialysis assay. For accurate measurement of FT_4, minimal adsorption of FT_4 to equilibrium dialysis membranes are required [93,98,99]. Potential leakage of binding proteins through dialysis tubing needs to be avoided and evaluated. Separation of FT_4 from bound T_4 needs to be done with minimal disturbance of the free, bound equilibrium [98].

3.1.1 Factors that may impair the validity of equilibrium dialysis methods

3.1.1.1 Temperature and pH

It has been shown that the equilibrium between bound thyroxine and the free thyroxine is dependent on temperature, more specifically the association constant for the binding of thyroxine to TBG decreases when the temperature rises, a temperature increase from 20 to 37 °C can lead to a doubling in FT_4 concentration [100]. The assay used needs to reflect the *in vivo*

concentration of FT_4 and FT_3, which implies that equilibrium dialysis and ultrafiltration need to be performed at 37 °C.

pH influences the equilibrium between bound and free T_4. A 0.1 pH unit deviation from 7.40 results in an error in FT_4 concentrations between 3% and 5% [101,102]. Separation between bound and free T_4 is therefore often done at the physiological pH of 7.4. Equilibrium dialysis methods often include a step to adjust serum pH to 7.4 [96]. This adjustment to normal physiological pH needs careful consideration in patients with acid–base disturbances where this adjustment may result in a measured FT_4 concentration different from the true *in vivo* FT_4 concentration.

3.1.1.2 Dilution and the effect of drugs and other competitive inhibitors

An important limitation of equilibrium dialysis is the dilution step employed that may disturb the equilibrium between bound and free fractions. Dilution may result in an underestimation of true FT_4 in the presence of low-affinity binding protein inhibitors [103]. On dilution, the effect of a dialyzable competitor (i.e., drugs, FFAs, or disease-specific endogenous compounds in nonthyroidal illness) will be underestimated with the highest error in those assays with the highest sample dilution [93]. Ideally, dialysis methods should be performed with as little dilution as possible [93]. As with direct analogue immunoassays for the measurement of FT_4 that underestimate FT_4 in nonthyroidal illness, equilibrium dialysis methods which use a diluted serum sample will also underestimate FT_4 concentration [103,104]. No equilibrium dialysis method can be performed without dilution, as the buffer volume should be included in the dilution factor [11].

3.1.1.3 FFAs/heparin artifacts

In patients treated with heparin, the enhanced lipase activity triggered by heparin may increase the FFA concentration displacing T_4 from its binding proteins [76]. The equilibrium dialysis methods are particularly prone to this artifact due to the long incubation step performed at 37 °C [93] (usually 17–24 h).

3.1.1.4 Adequate equilibrium

It is important that adequate time is allowed to reach equilibrium. Adequate equilibrium typically requires at least 17 h.

3.2. Ultrafiltration

Compared to equilibrium dialysis, the ultrafiltration methods [24,25,105] are more amenable for use in clinical laboratories. The basic principle behind

ultrafiltration is that proteins due to their molecular size are unable to cross-over a semipermeable membrane. Centrifugation is used to generate enough force to allow small molecules to pass through the semipermeable membrane whereas proteins and protein-bound compounds are retained.

Potential advantages of ultrafiltration include shorter analysis time, less sample requirement, and better precision [25,106,107]. In general, the correlation between ultrafiltration and equilibrium dialysis method has been shown to be very good [25,108].

3.2.1 Factors that may impair the validity of ultrafiltration methods

3.2.1.1 Temperature and pH

Temperature at which ultrafiltration is performed affects FT_4 concentration. An increase in the temperature of ultrafiltration from 25 to 37 °C results in a 1.5-fold increase in the concentrations of both FT_4 and FT_3 [32]. Ultrafiltration at 37 °C matches body temperature.

As no buffer is used before generation of the protein free serum, ultrafiltration is not susceptible to the potential problems associated with the use of the buffer solution required for equilibrium dialysis.

3.2.1.2 Adsorption and protein leakage

Adsorption of thyroxine to the ultrafiltration membrane and protein leakage through the ultrafiltration membrane needs to be evaluated and avoided [98]. Binding protein leakage during ultrafiltration may cause falsely increased FT_4 results. To reduce the chance of potential protein leakage, the ultrafiltration device selected and amount of centrifugal force used for ultrafiltration needs special consideration. While higher "*g*" values shorten the ultrafiltration process membranes can break when exposed to these conditions and the optimum centrifugal force selected needs careful evaluation.

A summary comparing ultrafiltration and equilibrium dialysis methods is provided in Table 4.2.

4. ISOTOPE DILUTION MASS SPECTROMETRY COUPLED TO PHYSICAL SEPARATION STEPS

LC–MS/MS methods identify the compound of interest by both retention time and mass-to-charge ratio of parent and fragmentation ions and therefore offer the advantage of greater analytical specificity and less analytical interference when compared to immunoassays [25]. In 2005, Soldin *et al.* described a method to quantify FT_4 using ultrafiltration followed by

Table 4.2 Comparison between ultrafiltration and equilibrium dialysis

	Ultrafiltration	Equilibrium dialysis
Buffer and dilution	Serum sample undergoes ultracentrifugation for separation of free from bound hormone before dilution	Serum samples dialyzed against dialysis buffer. Dilution may result in possible disturbance between bound and free hormone
Protein leakage/ adsorption	Possibility of protein leakage Requires careful evaluation of membrane used as well as centrifugal force	Possibility of adsorption to dialysis membrane
Sample volume requirement	400 µL [24]	200 µL [97] 1 mL [96]
Temperature	Should be performed at 37 °C	Should be performed at 37 °C
Time	Centrifugation for 30 min [24]	Between 17 and 20 h [97] Leads to possible free fatty acid generation
Total CV	4.1% and 6.6% [25]	3.95–7.48% [97] CV: 5.6% [96]
Limit of quantitation	6.3 pmol/L [105]	1.3 pmol/L [97] 1.3 pmol/L [96]

LC–MS/MS [25]. The method was further improved by use of a more sensitive mass spectrometer, the use of a different column and centrifugation at 37 °C [24,105].

Although LC–MS/MS methods are more specific than immunoassays, it is important to realize that differences in separation techniques used (equilibrium dialysis or ultrafiltration) and differences in dilution, pH, and temperature may still result in nonuniformity between mass spectrometry methods with some mass spectrometry methods being superior in some clinical scenarios compared to others.

4.1. Ion suppression

A factor to consider when evaluating mass spectrometry methods for the measurement of FT_4 and FT_3 is the effect of ion suppression. Ion suppression can be minimized by enhancing specimen cleanup, utilizing gradients to separate interferants from the analyte of interest and assessing internal

standard peak heights which should remain more or less constant between samples [22].

4.2. Derivatization versus nonderivatization

Derivatization may improve the analytical sensitivity of LC–MS/MS methods. However, it also adds an extra step that makes it less convenient for adoption in routine clinical laboratories. The method becomes more labor intensive, requiring more technical intervention and increasing both analysis time and imprecision. With the improved analytical sensitivity of modern mass spectrometers, derivatization is no longer a requirement for the measurement of thyroxine and triiodothyronine [22].

4.3. Potential advantages of physical separation methods followed by LC–MS/MS detection

Measurement by LC–MS/MS is accurate, precise, and more specific than immunoassays. Physical separation methods allow for the reliable measurement of FT_4 and FT_3 in any of the conditions that may result in changes in binding protein concentration. These include pregnancy and nonthyroidal illness.

Another advantage of measurement by LC–MS/MS is that it allows for the potential measurement of FT_4, FT_3, rFT_3, and FT_2 in the same analytical run. This can provide a more complete view of free thyroid hormone status and may be of benefit both in research and in certain clinical conditions such as pregnancy and nonthyroidal illness.

4.4. Potential disadvantages of physical separation methods followed by LC–MS/MS detection

Currently, LC–MS/MS assays are not as automated as immunoassays. Because of this, it is more difficult for most routine laboratories to provide a 24-h service for the measurement of FT_4/FT_3 by LC–MS/MS and because of this turnaround time may not be as quick as with traditional immunoassays. Measurement by LC–MS/MS also requires an initial investment in instrumentation and training of staff.

5. STANDARDIZATION AND REFERENCE METHOD FOR THE MEASUREMENT OF FT_4

FT_4 results by immunoassay remain poorly standardized [35,109,110]. This difference between methods is even more pronounced in various clinical states such as critical illness, renal failure, and pregnancy [17] and remains

a huge problem for the clinician. A critical step in improving standardization between methods is the selection and validation of a reference measurement procedure. The selection of the reference method for the measurement of FT_4 requires careful consideration. Thyroxine is a clearly defined small molecule and proposed reference methods for the measurement of thyroxine exist.

A FT_4 method based on equilibrium dialysis followed by isotope dilution LC–MS/MS in the dialysate has been proposed as a potential candidate reference measurement procedure [96,109,111–113]. Equilibrium dialysis and ultrafiltration as separation step both have important uncertainties that need careful consideration [93]. Since FT_4 measurement in serum should reflect the *in vivo* free hormone concentration at equilibrium with protein-bound hormone, the reference measurement procedure should fulfill the premise of nondisturbance between the bound and free fraction of thyroxine [93,111].

6. EVALUATION OF ASSAYS FOR CLINICAL USE

The measurement of FT_4 is used in various clinical conditions to both diagnose disease and guide treatment. Ideally, methods should be tested in clinical samples with particular attention to those conditions that challenge the analytical validity of the assay. This includes the evaluation of assays in pregnancy and patients with nonthyroidal illness and renal disease. The effect of drugs on assay performance also needs to be evaluated; unfortunately, this is seldom done by manufacturers [35]. There needs to be a good relationship between FT_4 and log TSH. This condition is met by both equilibrium dialysis and ultrafiltration tandem mass spectrometric methods. In our opinion, the FDA needs to critically assess this relationship before approving platform immunoassay FT_4 methods.

7. SOME CLINICAL EXAMPLES OF INACCURATE TT_3, FT_4, AND FT_3 MEASUREMENT BY IMMUNOASSAYS

Poor correlation between FT_4 measured by immunoassay and log TSH suggests that TT_4/FT_4 and TT_3/FT_3 may need to be evaluated by mass spectrometric methods in patients complaining of symptoms consistent with hypothyroidism but with apparent normal immunoassay measurements for TT_3, TT_4, FT_4, and TSH. Among these patients, we have recently uncovered several with normal immunoassay TT_4/FT_4 and TT_3/FT_3 but with TT_4/FT_4 and/or TT_3/FT_3 below the 2.5th percentile when measured by

mass spectrometry. Treatment with a combination of T_4 and T_3 normalized the thyroid parameters when measured by mass spectrometry and alleviated the symptoms of complaint that had brought these individuals to our attention. We also identified a patient with Kaposi Sarcoma with low FT_4 and FT_3 as measured by immunoassay but with normal TSH. FT_4 and FT_3 measured by ultrafiltration followed by LC–MS/MS were normal. Of note is that this patient had a TBG concentration well below the normal reference range and in our opinion, the immunoassay was unable to accurately measure free hormone concentration in a setting of this abnormal binding protein concentration.

It has been shown that patients with certain deiodinase polymorphisms have lower T_3 levels [114,115]. Reliable measurement requires a good method not only for FT_4 and FT_3 but also for TT_4 and TT_3 [37]. Patients with deiodinase deficiencies are now being diagnosed more frequently in our laboratory because the mass spectrometric TT_3 assay is far superior to immunoassays for TT_3 which often give falsely elevated (apparently normal) results. In these individuals, the mass spectrometric TT_3 is low. The identification of these patients by accurate T_3 assays guides therapy that is more appropriate and leads to alleviation of symptoms.

Working closely with our endocrine faculty, we have noticed that when the TSH's are high the direct analogue IA's will often have normal FT_4's. Measurement of FT_4 by either equilibrium dialysis or ultrafiltration mass spectrometric methods will provide the expected low results on these samples. As clinical awareness of limitations of immunoassays increases more patients with inaccurate FT_4 and FT_3 results by immunoassay will be identified.

8. CONCLUSION

Measurement of FT_4 and FT_3 by immunoassay in certain population groups, including patients with binding protein abnormalities, pregnancy, and nonthyroidal illness remain suboptimal. Unfortunately, these FT_4 assays do not correlate well with log TSH, a very important requirement. Physicians need to be educated about the limitations of current routinely available immunoassay FT_4 and FT_3 methods.

FT_4/FT_3 by physical separation methods, such as ultrafiltration, followed by LC–MS/MS are a significant improvement over direct analogue immunoassays and provide a viable alternative to current immunoassays. They have already been proven to be superior in many clinical situations.

A huge advantage is that these new mass spectrometric FT_4 measurements correlate well with log TSH [23–26,28,29,32,44,116].

In larger laboratories, measurement of all FT_4 samples by mass spectrometry may be prohibitive due to high sample volumes. A potential compromise is to use TSH levels to help in selecting those samples which are most likely to benefit from a mass spectrometric approach. Individuals with TSH's below the 10th percentile or above the 90th percentile are most at risk of having hyper and hypothyroidism, respectively. We recommend that FT_4 measurement by LC–MS/MS be done for these specimens. Clinicians should be aware of the potential limitations of immunoassays and, if interference is suspected, be able to order measurement of FT_4 and FT_3 by a physical separation method.

Finally, the need for accurate (non-immunoassay) measurement of TT_3 and FT_3 is growing as we become more aware of the importance of deiodinase deficiencies and the clinical conditions that impact synthesis and conversion of T_4 to T_3.

ACKNOWLEDGMENT

This work was partially supported by the Intramural Research Program of the NIH.

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