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Analysis of free, unbound thyroid hormones by liquid chromatography-tandem mass spectrometry: A mini-review of the medical rationale and analytical methods

Alexander B. Westbye[1,2] | Finn Erik Aas[1,2] | Oskar Kelp[1,2] | Louise K. Dahll[1,2] | Per M. Thorsby[1,2,3]

[1]Hormone Laboratory, Department of Medical Biochemistry, Oslo University Hospital, Oslo, Norway

[2]Biochemical Endocrinology and Metabolism Research Group, Oslo University Hospital, Oslo, Norway

[3]Institute of Clinical Medicine, University of Oslo, Oslo, Norway

Correspondence
Alexander B. Westbye, Hormone Laboratory, Department of Medical Biochemistry, Oslo University Hospital, Aker, Oslo, Norway.
Email: alwest@ous-hf.no

Abstract

Measurement of hormones is important for the diagnosis and management of endocrine diseases. The thyroid hormones thyroxine (T4) and triiodothyronine (T3) are among the most commonly measured hormones in clinical laboratories, and it is the concentration of free (not bound to proteins) thyroid hormones that is clinically most relevant. Free thyroid hormones are commonly measured using automated immunoassays, however, these are known to produce erroneous results due to interferences for some patients. Measurement of free thyroid hormones using equilibrium dialysis or ultrafiltration combined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is considered a more accurate and robust method for free thyroid hormone analysis and overcomes many of the limitations of immunoassays. However, LC-MS/MS-based methods are often considered too technically difficult and not amendable to high throughput by clinical chemists and are not offered by many clinical laboratories. This mini-review aims to make it easier for clinical laboratories to implement LC-MS/MS-based measurement of free thyroid hormones. It describes the medical rationale for measuring free thyroid hormones, the benefits of LC-MS/MS-based methods with respect to interferences affecting immunoassay-based methods and physical separation methods. This mini-review highlights important parameters for ultrafiltration and equilibrium dialysis to obtain physiologically relevant free thyroid hormone concentrations and focuses on methods and devices used in clinical chemistry.

1 | INTRODUCTION

Hormones are signalling molecules produced by endocrine glands and released into the blood circulation. The hormones bind receptors in a variety of tissues and thereby regulate cellular functions.[1] Disturbance in hormone concentrations underlies numerous endocrine diseases, and the precise measurement of hormone concentrations in blood

Abbreviations: TH, thyroid hormone; T4, Thyroxine; T3, triiodothyronine; FT4, free T4; FT3, free T3; rT3, reverse T3; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem MS; TSH, thyroid-stimulating hormone; TBG, thyroxin-binding globulin; FDH, familial dysalbuminemic hyperthyroxinemia; MWCO, molecular-weight cutoff; SPE, solid phase extraction; RED, Rapid Equilibrium Dialysis; TG, triglycerides.

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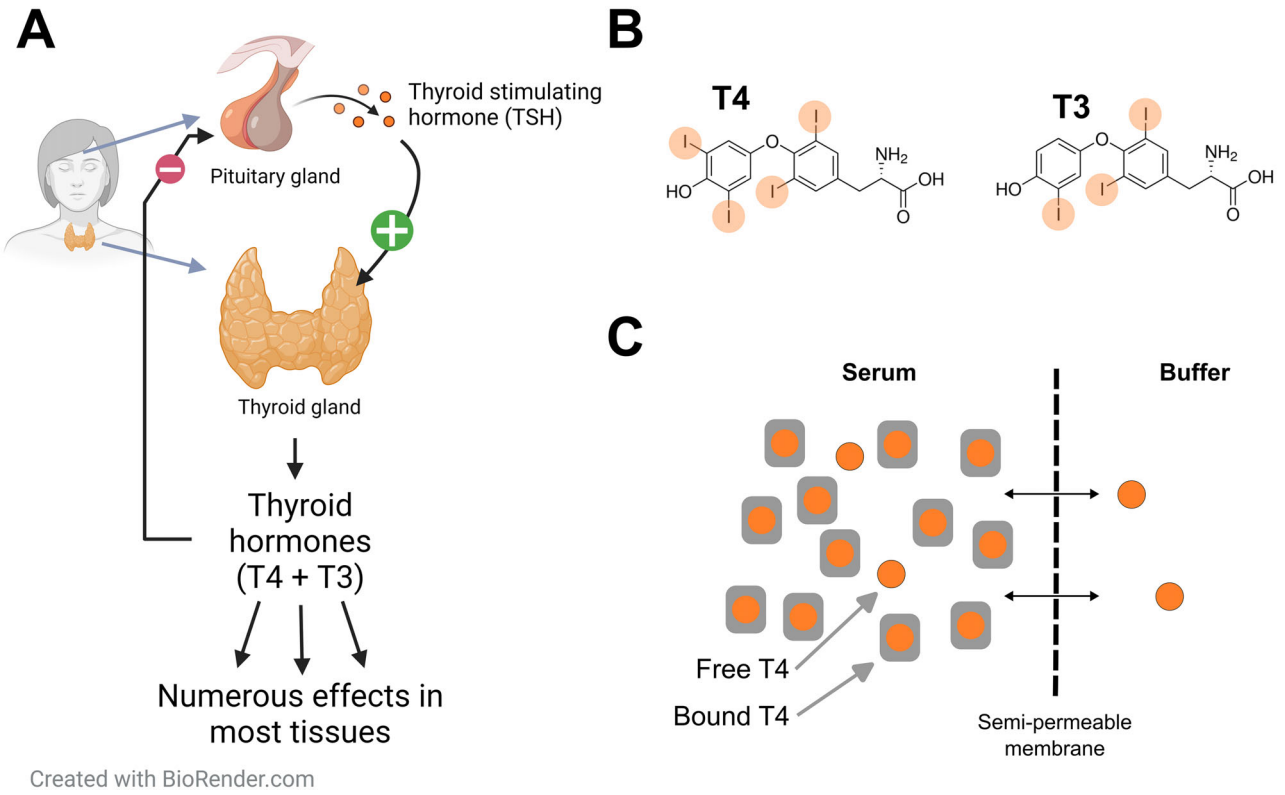

FIGURE 1 Thyroid hormones. (A) The thyroid hormones thyroxine (T4) and triiodothyronine (T3) are produced by the thyroid gland. Their production is stimulated by thyroid stimulating hormone (TSH), released by the pituitary gland. T4 and T3 exert a negative feed-back regulation on TSH production. (B) The chemical structure of T4 and T3 (iodines highlighted). (C) Illustration of free T4 and the principle of equilibrium dialysis. More than 99% of T4 (orange) in blood plasma/serum is bound to binding proteins (grey). A very small percentage of T4 is free. This fraction can cross a semi-permeable membrane during dialysis, establishing an equilibrium between free T4 in serum and T4 in the protein-free buffer.

plasma or serum is essential for the diagnosis and management of endocrine diseases.[2]

Thyroid hormones (THs) are produced by the thyroid gland and are unique by containing iodine (Figure 1A,B). THs are central to the development and wellbeing of humans and other vertebrates, including correct growth and development in utero and after birth. Furthermore, they are important for normal cognitive functions and regulation of metabolism.[3–5] THs refer to two compounds: Thyroxine (T4), a prohormone present at a relatively high concentration in circulation, and the active hormone triiodothyronine (T3) (Figure 1B). THs act on most tissues in the body, and the majority of T3 production occurs in the tissues through enzymatic deiodination of T4. Furthermore, the production of THs is regulated by thyroid stimulating hormone (TSH) (Figure 1A).[6]

Whenever clinicians suspect thyroid disease, measurement of TSH is the primary analytical parameter to investigate the hypothalamus-pituitary-thyroid axis. If the TSH test indicates disease or the clinician suspects dysregulation of TH production, follow-up tests involve measurement of free THs. Serum-free T4 concentration is among the most commonly performed clinical diagnostic test and is essential whenever clinicians suspect that the thyroid gland produces too little or too much THs (for in-depth reviews of clinical measurement of free TH[7,8]). Thyroid diseases are prevalent in the global population: Hypothyroidism, a disease caused by insufficient concentrations of circulating THs, is by far the most common thyroid disease and affects up to 10% of the middle-aged female population in developed countries. The prevalence of overt hypothyroidism globally is estimated at 1%–2% in iodine-sufficient areas. In Europe, prevalence ranges from 0.2% to 5.3% and in the USA 0.3% to 3.7%.[9] Treatment involves oral intake of THs, usually levothyroxine (synthetic T4), and levothyroxine was reported as the second most prescribed medication in the US.[10] In contrast, in hyperthyroidism there is an excess of circulating THs. The prevalence of overt hyperthyroidism was reported to range from 0.2% to 1.3%.[9] Both hypo- and hyperthyroidism are often caused by autoimmunity and are more common among women than men.[9]

2 | THE CONCENTRATION OF FREE HORMONES IS CLINICALLY IMPORTANT

Measurement of T4 (and T3) for clinical purposes is not straight forward, because the hormones interact with binding proteins (Figure 1C): more than 99.9% of T4 present in blood circulates bound to three binding proteins: thyroxine-binding globulin (TBG; binds ~70%), transthyretin (20%) and albumin (10%). According to the free hormone hypothesis (reviewed[11]) it is only the non-bound, free fractions of T4 (FT4) and T3 (FT3) that exert a biological activity and therefore are the

most relevant fractions to measure. Measurement of total THs is typically less clinically informative than free THs, as several conditions and pharmaceutical medications alter the total concentrations of THs in blood, without affecting the free concentrations. In particular, changes in TBG can have profound effects on total TH, and several common conditions (e.g. pregnancy) and medications (oral estrogens, used in some birth control pills) increase TBG and thereby total TH. In contrast, other hormones (androgens and glucocorticoids) reduce TBG.[12] Formulas have been devised to calculate clinically useful indexes for free hormones that account for the concentration of binding proteins; however, these ratios or indexes do not perform well for patients with large deviations in binding proteins.[8,13] It is therefore important to be able to measure the concentration of free THs.

Concentrations of free THs in the blood are very low, typically in the ranges ~15–28 and ~4–10 picomol/L (a picomol is 10^{-12} mol) for FT4 and FT3, respectively, in healthy individuals[14] (Table 1) and measurement methods therefore need to be sensitive. Furthermore, clinical routine tests for THs typically have to be high throughput and be available at a relatively low cost. Automated immunoassays are today the predominant technology used to measure free TH because they are affordable, easy to operate and offer fast analysis and turnaround times. However, these immunoassays are prone to interferences for some patients, resulting in erroneous results.[15,16] Many of these interference errors are absent if free hormones are analyzed by methods that use dialysis or ultrafiltration followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).[17] LC-MS/MS-based methods are by many considered the "gold standard" for clinical measurement of free TH and form the basis of the proposed reference methods for free TH measurement.[14,18–20] However, most clinical laboratories consider LC-MS/MS-based methods to be too laborious, technically difficult and too expensive for routine analysis of free THs, and the method is not widely used. Recently, the application of 96-well plate-based microdialysis[21] has greatly enhanced the throughput and some specialized clinical labs (e.g. Mayo Clinic Laboratories and Labcorp) offer analysis of FT3 and/or FT4 by equilibrium dialysis LC-MS/MS. Below we outline immunoassay-based methods for free TH measurement and some of their limitations, and how free hormones can be measured using LC-MS/MS to overcome most of these limitations.

3 | MEASUREMENT OF FREE THs BY IMMUNOASSAYS AND LIMITATIONS DUE TO INTERFERENCES

Multiple diagnostic immunoassays are available to measure FT4 and FT3, and some of the most widely used clinical assays are automated immunoassays that run on clinical chemistry analyzer systems. These platforms offer high sensitivity and rapid measurement time with very little hands-on time per sample.[8] Many instruments can be set up to perform multiple different clinical analyses and often run as "random access" analyzers, meaning that samples do not need to be analyzed in any specific order as opposed to analysis-specific batches (as is done for LC-MS) making it possible to rapidly analyze a sample if needed. Immunoassays are furthermore generally considered cost-efficient compared to LC-MS/MS-based diagnostic methods.

Central to immunoassays is the use of antibodies to detect the analyte (Figure 2). Immunoassay methods that measure free TH are typically "one-step" competitive assays (e.g. Roche Cobas and Siemens Immulite platforms) that utilize labelled TH analogues that prevent binding to the TH-specific binding proteins and compete with free hormones for a limited number of antibody binding sites. Alternatively, "two-step" assays (eg. Abbott Alinity) employ antibodies to bind (capture) a (very small) fraction of the free hormones to a solid support. Unbound hormones and proteins are washed away in an additional washing step before a labelled hormone-analogue is added in a second step. The labelled analogue then binds to the unoccupied antibodies (a procedure referred to as "back-titration").[22]

The majority of immunoassays for FT4 and FT3 reportedly perform well for most patients,[14,22] however most assays measure a lower concentration (negative bias) than methods that involve physical separation of free and bound TH (e.g. equilibrium dialysis). A practical clinical issue is that the free hormone concentration measured for the same sample differs between platforms,[14,23] requiring comparison to reference ranges established on the specific platform. This can complicate the longitudinal monitoring of patients if the laboratory changes its method, or if patient samples are analyzed at more than one laboratory (active efforts to harmonize or standardize measurements of THs are ongoing.[24])

Immunoassays are in general known to be prone to interferences that can give falsely increased or reduced laboratory results for certain patients.[16,22,25,26] These include unintended antibody cross-reactions with a compound similar to the analyte or the presence of endogenous antibodies (e.g. human anti-mouse antibodies or rheumatoid factors present in the sample). These endogenous antibodies can interfere by binding components of the assay, producing erroneously high or low results.

Patients with autoimmune disease, including autoimmune thyroid disease, can have antibodies against THs. The percentage of patients with autoantibodies against T4 or T3 varies and was reported to be around 20% for Hashimoto's thyroiditis and up to 46% of the patients with Grave's disease. These can be transient and some patient's antibodies can interfere with TH immunoassays.[16,27–29] A recent study concluded that for 9% of the autoantibody-positive samples investigated, a commonly used one-step immunoassay platform produced erroneous results.[27] In addition, interference due to biotin (Vitamin B7/B8; caused by ingestion of high doses of biotin) is an emerging issue because many immunoassays employ a biotin-streptavidin complex to bind antibodies to surfaces.[30] Furthermore, we recently reported that endogenous streptavidin-antibodies that cause interference in thyroid analyses were more common than previously suspected and may affect about 0.4% of all samples for FT3 analysis.[15]

Binding protein concentrations and variants can also affect the accuracy of measured free TH concentrations. Several authors have concluded that some immunoassays are not well suited to measure THs during conditions of altered TBG concentrations including pregnancy

TABLE 1 Selected published liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods to measure free thyroid hormones (THs) and their validation results.

	Yue (2008)[21]	Refetoff (2020)[35]	Ting (2021)[72],[d]	Jansen (2023)[18]	"Soldin method"[a]	Hernández (2021)[61]
Analysis	FT4 & FT3	FT4	FT4 & FT3	FT4	FT4 & FT3	FT4
Separation mode	Equilibrium dialysis	Equilibrium dialysis	Equilibrium dialysis	Equilibrium dialysis	Ultrafiltration	Ultrafiltration
Device and membrane/filter (Producer)	DispoEquilibrium Dialyzer (Harvard Apparatus)	DispoEquilibrium Dialyzer (Harvard Apparatus)	Rapid Equilibrium Dialysis (ThermoFisher Scientific)	Micro-Equilibrium Dialyzer System (Harvard Apparatus)	Centrifree (Millipore)	Centrifree (Millipore)
MWCO	5 kDa	10 kDa	?	5 kDa	30 kDa	30 kDa
Sample format	96-well plate format	96-well plate format	48-samples (96-well plate format)	Single sample	Single sample	Single sample
Temperature, °C	37	37	?	37	37 (25[b])	26 (!)
Serum volume	200 µl	200 µl	?	1 ml	400 µl	500 µl
Sample cleanup	Online SPE	None	?	LLE[c]	Online SPE	None
Lower LOQ or lower measuring range, pM	1.3 (FT4) 1.5 (FT3)	2.6 (FT4)	?	1.39 (FT4)	6.3 (FT4) 1.5 (FT3)	3.9 (FT4)
Upper LOQ or upper measuring range, pM	> 500 pM (ULOQ)	164 (FT4)	?	?	63 (FT4) 38.5 (FT3)	25.8 (FT4)
CV, within run	2.1%–5.6% (FT4) 3.7%–6.14% (FT3)	7.1%–9.1% (FT4)	?	1.0%–3.7% (FT4)	4.1%–6.6% (FT4)< 9% (FT3)	< 7.2 (FT4)
CV, between run or total	2.5%–5.5% (FT4) 5.5%–10.3% (FT3)	7.3%–9.4% (FT4)	?	3.1%–3.9% (FT4)	< 7% (FT4)< 9% (FT3)	< 12.6% (FT4)
Normal FT4 ranges adults, pM	16.5%–28.6	11.6–28.3	–	–	17.4–30.9	–
Normal FT3 ranges adults, pM	5.56%–10.4	–	–	–	2.3–9.5	–
Other established ranges or clinical populations studied	Pregnancy 2nd trimester (week specific)	Ranges for children < = 2 years and 3–17 years; FDH population	FDH patients	–	Age and gender-specific intervals (1 month–18 years; Soldin 2009)	Pregnancy 1st trimester
Comparison with other methods	Yes	Yes	Yes	Yes	Yes	Yes

[a]«Soldin method» refers to the ultrafiltration LC-MS/MS method initially published by Soldin (2005),[54] and modified in subsequent publications by Gu (2007),[60] Soldin (2009),[67] Gounden (2014)[59] and van Deventer (2011).[33] Most recently available information used in the table.

[b]Ultrafiltration was initially performed at 25°C, but later changed to 37°C. Discussed in Soldin (2009).[67]

[c]Other sample preparation procedures were also described and reported to be suitable.

[d]Ting (2021)[72] does not provide sufficient information (e.g. dialysis and LC-MS/MS parameters) to allow the reproduction of the method.

Abbreviation: CV, coefficient of variation; FDH, Familial dysalbuminemic hyperthyroxinemia; LLE, liquid-liquid extraction; LOQ, limit of quantitation; SPE, solid-phase extraction.

A dash (–) indicate not covered/reported in publication.

A "?" indicate important method-specific information or validation results that was not found in the publication(s).

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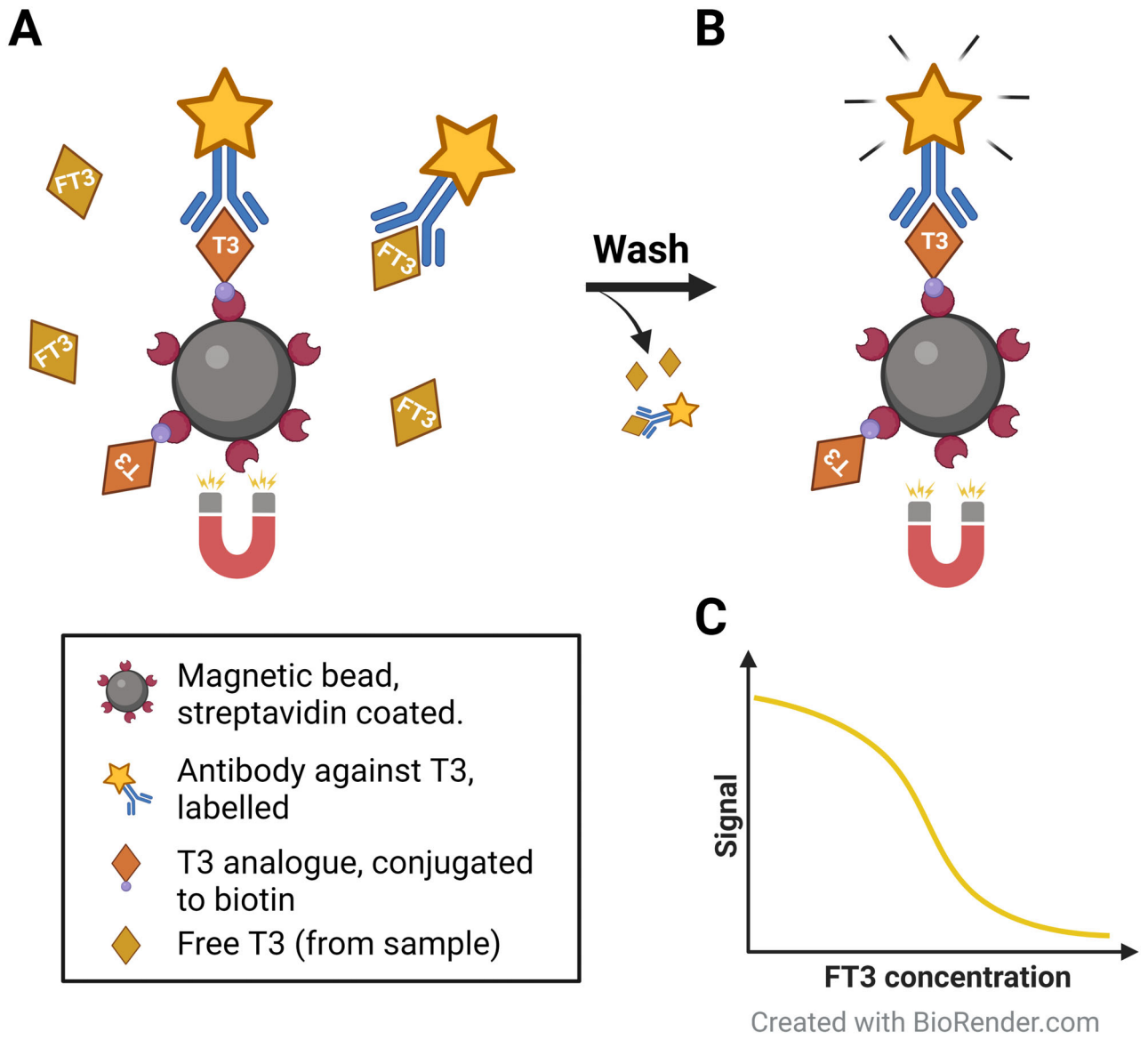

FIGURE 2 Immunoassay-based detection. Example of immunoassay-based "one-step" measurement of free T3 (the Roche competitive FT3 method). (A) A limited amount of labelled antibody binds either free T3 (from serum) or a biotin-conjugated T3-analogue. The biotin-conjugated analogue binds to streptavidin-coated magnetic beads. (B) Components not bound to the beads are washed away. Subsequent excitement of the label produces a signal that is measured. (C) The signal is inversely-proportional to the original amount of free T3 present in the sample.

(increased TBG) and conditions of low TBG.[23,31–33] Several investigators have reported that multiple immunoassays measure falsely elevated FT4 concentrations for patients with familial dysalbuminemic hyperthyroxinemia (FDH).[34,35] While generally considered a rare condition, the prevalence of FDH was reported to be ~1% in a Hispanic population.[36] The cause of FDH is genetic variants of the binding protein albumin that leads to increased affinity for T4, resulting in greatly elevated total T4-concentrations.[37] However, FDH patients are clinically euthyroid (i.e. "thyroid healthy") with normal TSH-concentrations and do not need any treatment. The enhanced binding of labelled TH-analogues to albumin results in falsely elevated FT4 and FT3 when measured with many immunoassays, erroneously indicating TH overproduction. As a result, FDH patients are often misdiagnosed and some have received inappropriate treatment (e.g. thyroid gland ablation). In contrast, when FT4 was measured for FDH-patients using dialysis LC-MS/MS the values were similar to non-affected individuals and generally within the reference range.[35,38]

4 | MEASURING FREE THs USING LC-MS/MS

4.1 | Overview of TH measurement by LC-MS/MS

LC-MS/MS can provide highly specific and sometimes sensitive analyses. In LC-MS/MS analyses, compounds are first separated chromatographically on an analytical column and then analyzed by a tandem mass spectrometer (for a handbook on LC and an advanced textbook on MS[39,40]). LC-MS/MS is becoming an increasingly impor-

tant and common analytical tool in clinical biochemistry, including endocrinology.[41,42] Modern triple quadrupole MS are sufficiently sensitive that THs and related metabolites can readily be analyzed by LC-MS/MS,[43,44] including the very low free TH (FT4 and FT3) concentrations.[45] Table 1 summarizes several publications that measure free THs by LC-MS/MS with an emphasis on publications that either use different technology to separate free from bound hormones and/or that provide key validation results and published reference intervals.

In general, THs (including their structural isomers and metabolites) separate well on C18-columns using water:methanol gradients and their retention times increase with increasing number of iodines.[43] THs ionize well using electrospray ionization and fragment well using collision-induced dissociation. Chemical derivatization is therefore not typically employed; however, butyl ester-derivatization has been investigated for tissue extracts.[46] It is noteworthy that serum contains the inactive compound reverse-T3 (rT3), a structural isomer of T3 that has the exact same mass and a very similar fragmentation pattern. rT3 is produced from T4 under certain conditions, including severe illness ('Non-Thyroidal Illness Syndrome').[6] Methods should chromatographically separate these two compounds, although the concentration of free rT3 is very low[47,48] and in our experience generally below quantitative limits for LC-MS/MS.

For LC-MS/MS measurements in a clinical setting, isotope dilution[49,50] and absolute quantitation using relative peak area to give concentration values (e.g. pM) of high quality is typically wanted or required. This involves the addition of a known concentration of an isotopologue (i.e. a variant of the measurand that incorporates stable isotope(s), such as deuterium, ^{13}C or ^{15}N). The isotopologue behaves very similarly to the measurand and is used to correct for loss (eg. adsorption) or concentration/dilution that may occur during sample preparation and to control for sample-specific ionization-effects during LC-MS/MS analysis (for an advanced textbook on isotope dilution in MS[50]). THs and metabolites (e.g. reverse-T3) labelled with stable isotopes are commercially available (e.g. Thyroxine-$^{13}C_6$ from Sigma Aldrich).

In certain extreme cases, FT4 concentrations can exceed the measuring range of immunoassays. It is not possible to dilute the serum to bring the concentrations within the measurement range, due to the equilibrium with bound T4. LC-MS/MS methods offer a substantially larger dynamic measurement range than immunoassays, and this was reported to be useful in one clinical case of extreme thyrotoxicosis.[51]

Typical sample preparation methods utilized for LC-MS/MS analyses of lipophilic compounds involve extraction of the compounds of interest from the sample using organic solvents. Direct organic extraction of serum is not suited for the study of free hormones (or free drugs), as this extracts both free and protein-bound hormones. An initial physical separation step that generates a sample fraction that is devoid of binding proteins and has a concentration of hormones equal to or very similar to the free hormone concentration in serum is therefore required. Ultrafiltration or equilibrium dialysis are two methods that utilize a semipermeable membrane or filter that prevents proteins and other large molecules or particles from passing through while allowing small molecules (e.g. the free THs) to cross, and are described in more detail below. Physical separation using size exclusion chromatography (e.g. Sephadex columns) has been investigated in the past,[52] but is not covered in this review. We note that an in-depth review covering the measurement of free hormones using LC-MS/MS was recently published by Kushnir et al.[45] and that there exists a guideline for the measurement of free hormones published by the Clinical and Laboratory Standards Institute (CLSI).[53]

Clinical laboratories should be aware that because the free TH concentrations measured by LC-MS/MS are substantially increased compared to immunoassay results,[14,23] the adoption of an LC-MS/MS analysis method will involve establishing or at least verifying clinical reference ranges, and that these differ markedly from most existing immunoassay-based ranges that the clinicians may be familiar with. This may cause immediate confusion in the medical community that uses the test results unless this difference is properly communicated.

4.2 | Free hormones obtained by ultrafiltration: possible, but not ideal?

Soldin et al.[54] pioneered the use of ultrafiltration and isotope dilution LC-MS/MS for FT4 analysis. However, we are not aware that any clinical laboratories offer an ultrafiltration-based method as a routine analysis and some concerns regarding the use of ultrafiltration for free TH measurement have been raised (see below).

Ultrafiltration employs a filter membrane with very small pore sizes that prevent large molecules (eg. proteins) and particulates from crossing the filter barrier while allowing small molecules to cross. Laboratory filters are typically fitted in single-sample devices that employ a centrifuge to generate force to move the liquid through the filter, but some 96 well-format devices (e.g. Pall AcroPrep) are also available. Ultrafiltration membranes for life science applications are typically characterized using molecular-weight cutoff (MWCO) based on the size (in Daltons) where at least 90% of the molecules (often a protein, but sometimes a polymer) are prevented from crossing the membrane.[55]

Several ultrafiltration devices are commercially available, usually with a selection of membrane-materials and MWCOs ranging from ~2 to several hundred kDa. The choice of ultrafiltration device appears to influence the measured free TH concentration and several devices were reported to leak TH-binding proteins across the filter, resulting in erroneously high free TH concentrations.[56–58] An ultrafiltration device that has been employed by several authors (Table 1) to obtain free THs is the Centrifree Ultrafiltration Device (30 kDa MWCO, Millipore), a disposable centrifugal filter.[51,59–62] Tanoue et al.[58] compared this device to two other 30 kDa MWCO ultrafiltration devices (Amicon Ultra and Nanosep Omega) for the measurement of free TH in bovine serum and concluded that Centrifree had superior performance in their setup. We note that Tikanoja reported in 1990[63] that Centrifree (and several other ultrafiltration devices) leaked sufficient albumin (0.009%) to not be suitable for FT4 measurement; however, the device may have been improved since. Furthermore, during the initial review of this

paper, an anonymous peer reviewer stated that both the Centrifree and Pall AcroPrep plates were unable to produce robust results in their experience (personal communication).

A commonly suggested "rule of thumb" for ultrafiltration is to select a membrane with an MWCO of one-half to one-third of the smallest protein that needs to be retained. Because the concentration of protein-bound T4 is very large compared to FT4, it is vital that binding proteins (and their bound THs) do not cross the filter. TBG and albumin are approximately twice the size of the 30 kDa MWCO: TBG is a 54 kDa monomer and serum albumin is a 67 kDa monomeric protein. TTR is a homotetramer of 55 kDa subunits.[64] A 30 kDa MWCO, therefore, appears to be at the border of these commonly employed "rules of thumb" with regard to the exclusion of TH-binding proteins in the filtrate.

Some authors have warned that ultrafiltration as a separation technique in general is not appropriate to study free THs, as the filtration process could produce falsely elevated free hormone concentrations.[65] Central to this argument is the "Donnan effect",[66] that stipulates that the progressive concentration of (charged) proteins in the retentate (the fluid that has not crossed the filter) may cause THs to be expelled to higher concentrations in the ultrafiltrate than the original free concentration.[65] However, we are not aware that this has been experimentally studied for THs.

Control of temperature and pH is essential when measuring FT4 because the equilibrium between free and bound T4 (and probably T3) is temperature and pH-dependent (serum pH can change upon exposure to atmospheric CO_2). Performing ultrafiltration at 25°C, as opposed to 37°C, lowers measured FT4 and FT3 by a factor of 1.5.[67] Ultrafiltration should therefore be performed at 37°C and maintenance of physiological pH (pH 7.4) should be ensured, if utilized.[8,68]

4.3 | Free hormones obtained by equilibrium dialysis

Equilibrium dialysis followed by LC-MS/MS is considered the most accurate method to quantify free THs and was the method chosen for the proposed reference method for measuring FT4.[20,69] Several different methods and validation results have been reported in recent times (Table 1).[18–21,35,70] Equilibrium dialysis of serum (plasma also appears acceptable) should be performed at 37°C and involve a (maximum of) 1:1 volume ratio of serum to a physiologically relevant dialysis buffer. Furthermore, pH must be maintained at 7.4 (at 37°C) to ensure the equilibrium between free and bound THs is not disturbed.[8,68]

During the dialysis process, THs (but not the binding proteins) in serum cross the semi-permeable membrane and a concentration gradient between free THs on the serum side of the (typically 5 or 10 kDa MWCO) and THs on the buffer side of the membrane (Figure 1C) is set up that eventually reaches equilibrium. Because of the vastly larger excess of bound to free T4 in serum, the resulting new equilibrium reached between bound and free T4 on the serum-side of the dialysis membrane is essentially identical to the original equilibrium in serum, and the FT4 concentration is essentially unaltered. Measurement of the "total" T4 concentration in the dialysis buffer compartment will then reflect the free T4 concentration in serum. In the past, available methods required samples to be dialyzed individually in single dialysis units, and the pH was manually adjusted to 7.4. In 2008, Yue et al.[21] reported a new method that increased throughput by combining 1) a 96-well format microdialyzer (DispoEquilibrium, 5 kDa MWCO), 2) a dialysis buffer that ensured that the serum reached the correct pH at 37°C after dialysis and 3) online solid-phase extraction (SPE). The authors used this method to report normal reference ranges (95% confidence intervals) for adults (FT4 12.8—22.2; FT3 3.62—6.75 pg/ml) and week-specific ranges during pregnancy (weeks 14–20).[21] This method was heralded as a major step towards the routine measurement of free THs by LC-MS/MS.[71]

There are at least two microdialysis-devices available for medium to high-throughput analysis that have been used to measure free THs: the 96-well DispoEquilibrium dialyzer (Harvard Apparatus) currently has a 10 kDa MWCO membrane and is designed for simultaneous assay of up to 96 samples (50–200 µl). Wells can be sealed by either plastic-strips or a pierceable self-sealing mat and has been used in several publications.[18,21,35] The Rapid Equilibrium Dialysis (RED, Thermo Scientific) is another "96-well format" microdialyzer, capable of dialyzing up to 48 samples in parallel and was used in an assay to investigate FT4 in FDH.[72] RED is available with 8 or 12 kDa MWCO membranes and is available with separate dialyzer inserts fitted into a baseplate. RED is marketed to reach equilibrium in ~ 4 h for some compounds. However, our preliminary testing indicated that the concentrations of THs had not reached equilibrium after 4 h. RED does require a slightly larger dilution than 1:1, ranging from 1:6 to 2:3 depending on sample volume (50–500 µl). We assume that a 2:3 dilution will likely not impact the measured free TH concentrations, at least for normal serum lacking TBG-binding drugs.[73] However, the reported leakage of plasma to the RED buffer compartment during dialysis[74] warrants caution, as a small amount of serum-contamination in the dialysate can greatly impact the measured "free" concentrations.

THs and binding proteins can adsorb to container walls, filters and membranes, including the dialysis and ultrafiltration units. For equilibrium dialysis, loss of free THs due to adsorption in both the serum and dialysate compartment is compensated for by increased dissociation of bound THs, and adsorption has been reported to cause at most a 2-3% lower estimation of FT4.[57] For subsequent sample processing, adsorption likely causes a net loss of THs; however, the addition of isotopically labelled internal standards should compensate for adsorptive losses resulting in little to no effect of adsorption on the measured concentration of FT4 and FT3.

4.4 | Clean-up of dialysate or filtrate prior to LC-MS/MS analysis

The dialysate or ultrafiltrate containing THs is very clean compared to serum, however, a sample preparation or clean-up step to

remove salts and buffering agents is generally advisable before LC-MS/MS analysis,[75] although some reported methods do not report any cleanup prior to injection of dialysate/ultrafiltrate onto the analytical column.[35,61,70] Presumably, a switching valve is utilized between the column and the ionization source of the MS, to at least reduce contamination of the MS by non-volatile dialysate/filtrate components (e.g. NaCl).

Online SPE (i.e. SPE performed using the (U)HPLC instrument on a designated column prior to analytical separation on an analytical column[76]) using C18-material has been used for both FT4 ultrafiltrate and dialysate.[21,54] Alternatively, offline (i.e. regular) extraction is possible, and both SPE, liquid-liquid extraction and supported liquid extraction have been used (e.g. comparison in[19]). Because THs have amine and carboxyl groups, they can be utilized in ion exchange-sample preparations. Jongejan et al.[43] recently reported the use of a mixed-mode strong anion exchange sorbent (Oasis MAX, Waters) for the purification of total THs from serum. We are currently employing this sorbent for the cleanup of dialysate with satisfactory results (Westbye et al., unpublished).

The addition of the isotopically labelled THs (internal standards) directly to serum is not feasible, as they would bind to the binding proteins (in an equilibrium), and their free concentration would be unknown. Instead, these are added to the dialysate or filtrate to correct for losses due to adsorption, solvent evaporation, etc. during subsequent sample preparation and to correct for matrix-effects (e.g. ion suppression) during LC-MS/MS analysis.[39,49]

4.5 | Analytical issues that also affect LC-MS/MS methods

Physical separation followed by LC-M/MS is considered the best alternative when the validity of an immunoassay result is questioned. However, certain interferences will also cause erroneous free TH measurements with LC-MS/MS-based methods.[17,77] Several drugs are known to influence measurements of free TH and several do this by altering the concentration of free TH in the test tube or during the measurement assay (i.e. after the blood sample is drawn from the patient).[73] Some medications (e.g. aspirin and furosemide) compete with T4 for binding to TBG. These drugs bind with a much lower affinity to TBG than T4 but can be present in blood in high concentrations relative to free THs. If dialysis is performed against a relatively large volume of buffer, an apparent change in free THs can occur due to the altered free drug equilibrium. This effect is also prominent in immunoassays that use large sample dilution.[73,77]

Administration of intravenous heparin (an anti-coagulant) to patients is known to cause major in vitro-artifacts by a complex mechanism if free THs are subsequently measured: Heparin-treatment induces mobilization of the enzyme lipoprotein lipase to blood. This enzyme is active in collected serum, where it acts on triglycerides (TGs; fats) that are present. The lipase cleaves the fatty acid tails of TGs, resulting in an increase in the concentration of free fatty acids (non-esterified fatty acids) upon storage. Because the lipase-generated free fatty-acids bind to TH-binding proteins and displace the bound THs, this causes an increased concentration of free THs in the test tube at the time of measurement compared to the original concentration prior to storage or incubation.[78,79] Because equilibrium dialysis involves extended incubation at physiological temperatures, such assays are likely highly prone to heparin-induced falsely elevated FT4 and FT3 results.

4.6 | Method validation

While LC-MS/MS methods are less prone to many interferences than immunoassays, proper method validation is important to ensure correct and robust results. Method validation needs to cover both the physical separations step (ultrafiltration or equilibrium dialysis) and the LC-MS/MS part of the method. Key method parameters include, but are not limited to quantitation limits, linearity and coefficients of variation. Population-specific reference ranges either need to be constructed or published reference ranges verified. It may also be advisable to investigate the consequences (if any) of incorrect sample matrices such as citrate or lithium-heparin plasma when the methods are validated for serum samples.

Several guidelines or standards exist on the topic of method validation, including the CLSI-62 guideline for LC-MS methods and specific articles about pitfalls[80] and general quality management considerations[81] for clinical LC-MS/MS analyses.

5 | SUMMARY AND OUTLOOK

Measurement of free THs, particularly FT4, is an important clinical analysis to diagnose thyroid disease and monitor patients.[8] Several automated immunoassay methods are available commercially and routinely used for the analysis of free THs. However, immunoassays are prone to interferences that produce false results in some patients. Furthermore, some of these interferences (TH autoantibodies) are especially prevalent among patients with thyroid disease.[16] Measurement of free THs using equilibrium dialysis or ultrafiltration followed by LC-MS/MS is considered less prone to interferences.[14] Today, LC-MS/MS appears as a feasible alternative method for routine analysis of free THs in specialized laboratories, at least for a subset of the samples. With current technology, it is probably not feasible or desirable that each clinical laboratory offers measurement of free THs using LC-MS/MS, or to use it in large screening studies (e.g. newborn screening). Rather, this could be offered as a service to the wider medical community in each country or state by one or more dedicated specialist ("reference") laboratories for endocrinology.

It is important to note that proper method validation and verification of or construction of reference intervals is important for any clinical method, including the measurement of free TH concentrations using LC-MS/MS.

AUTHOR CONTRIBUTIONS

Alexander B. Westbye: Conceptualization (equal), Writing—Original Draft (lead), Writing—Review & Editing (equal) and Visualization (lead). Finn Erik Aas: Conceptualization (equal) and Writing—Review & Editing (equal). Oskar Kelp: Writing—Review & Editing (equal). Louise K. Dahll1: Conceptualization (supporting), Writing—Review & Editing (equal) and Supervision (equal). Per M. Thorsby: Writing—Review & Editing (equal) and Supervision (equal).

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Alexander B. Westbye https://orcid.org/0000-0003-2619-4192
Louise K. Dahll https://orcid.org/0000-0002-3022-0755
Per M. Thorsby https://orcid.org/0000-0002-9615-1035

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