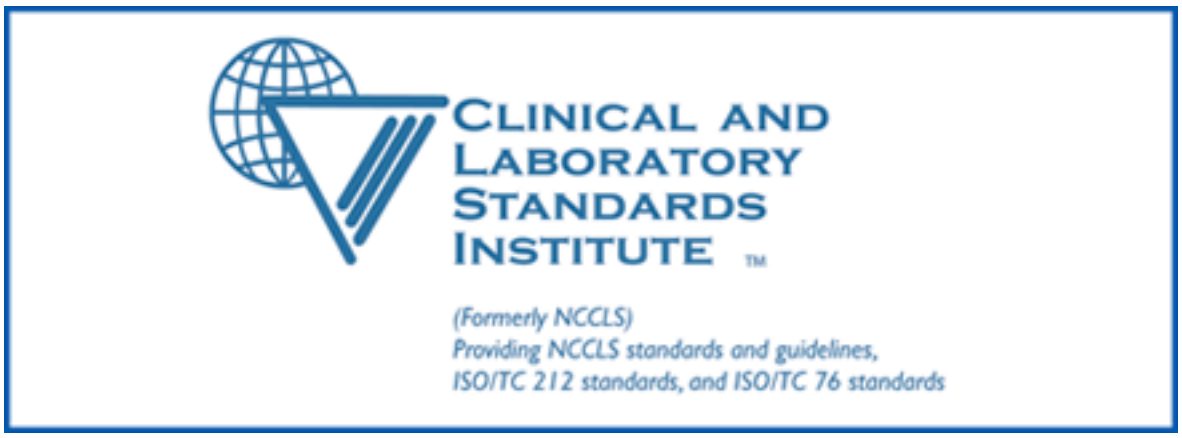
C45-A
Vol. 24 No. 31
Replaces C45-P
Vol. 24 No. 12

Measurement of Free Thyroid Hormones; Approved Guideline



This document addresses analytical and clinical validation of free (nonprotein-bound) thyroid hormone (FTH) measurement procedures.

A guideline for global application developed through the NCCLS consensus process.

NCCLS...

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C45-A
ISBN 1-56238-548-8
Volume 24 Number 31 ISSN 0273-3099

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Abstract

NCCLS document C45-A—*Measurement of Free Thyroid Hormones; Approved Guideline* is a guideline for free (nonprotein-bound) thyroid hormone (FTH) testing. The primary audience for this publication is personnel responsible for the development, manufacture, approval, and/or use of FTH measurement procedures. The guideline briefly discusses FTH measurement procedures with respect to design, factors confounding measurements, and specimen choice and collection, and addresses existing problems in definitions and specific nomenclature. However, it mainly emphasizes analytical and clinical validation of FTH measurement procedures. Assessment of analytical validity of an FTH measurement procedure not only implies demonstration of sufficient intrinsic quality or robustness against challenging factors, but also of metrological traceability to the Système International d'Unités (SI). In this respect, the guideline provides recommendations on the implementation of a reference measurement system in the development/validation process of *in vitro* diagnostic medical devices.

NCCLS. *Measurement of Free Thyroid Hormones; Approved Guideline*. NCCLS document C45-A (ISBN 1-56238-548-8). NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004.

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Suggested Citation

(NCCLS. *Measurement of Free Thyroid Hormones; Approved Guideline.* NCCLS document C45-A [ISBN 1-56238-548-8]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004.)

Proposed Guideline
March 2004

Approved Guideline
October 2004

ISBN 1-56238-548-8
ISSN 0273-3099

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Acknowledgement

This guideline was prepared by NCCLS, as part of a cooperative effort with IFCC to work toward the advancement and dissemination of laboratory standards on a worldwide basis. NCCLS gratefully acknowledges the participation of IFCC in this project.

Contents

Foreword

The measurement of free (nonprotein-bound) thyroid hormone (FTH) [free thyroxine (FT4) and free triiodothyronine (FT3)] concentrations in serum has been widely accepted to be clinically useful in differential diagnosis of thyroid disorders, monitoring therapeutic interventions, and follow-up of patients. However, along with the increasing use of FTH testing, confusion has arisen regarding definitions and specific nomenclature. Also, there is a continuing controversy regarding the validity of current routine measurement procedures. One major reason is that the design of some FTH routine measurement procedures insufficiently accounts for the Laws of Mass Action, and consequently lack a valid physicochemical basis. The lack of a generally accepted FTH reference measurement procedure for validation of routine measurement procedures additionally contributes to this controversy. Thus, there is an urgent need for clarifications and recommendations in these respects.

The validity of an FTH measurement procedure depends on two key requirements: (1) its capability to correctly determine serum FTH concentrations (i.e., with minimal analytical error and reflecting *in vivo* FTH); and (2) its capability to provide measurement results that are clinically useful. Therefore, designers/manufacturers urgently need minimum recommendations to assess/demonstrate both analytical and clinical validity of their measurement procedures. Furthermore, in an era of increasing efforts toward standardization of measurements in laboratory medicine, validity in the above terms extends to metrological traceability to a reference measurement system. Major drivers behind this concept have been the EC Directive on *In-Vitro* Diagnostic Medical Devices (98/79/EC) (IVD Directive) and supporting ISO/CEN standards. Consequently, the development of a reference measurement system for FTH measurement is of top priority. Following this, manufacturers of *in vitro* diagnostic medical devices shall be given guidance to implementing and demonstrating metrological traceability.

Therefore, the clarifications and recommendations given in this document are expected to benefit designers, manufacturers, regulators, and users of FTH measurement procedures.

Key Words

Accuracy, analytical/clinical validation, free thyroid hormone, reference measurement system, thyroxine, traceability, triiodothyronine, trueness

Measurement of Free Thyroid Hormones; Approved Guideline

1 Scope

This guideline, C45-A—*Measurement of Free Thyroid Hormones; Approved Guideline,* gives an overview of the pathophysiology of blood free thyroid hormones (FTH) and of the principles and limitations of current routine measurement procedures. Recommendations are included for specimen collection, stability, and storage. The main focus of the document is on the analytical and clinical validation of FTH measurement procedures and on their standardization by implementation of a reference measurement system. Specific recommendations for validating both analytical and clinical performance are provided. Direct equilibrium dialysis (ED) and direct ultrafiltration (UF) are proposed as candidate reference measurement procedures, and recommendations are provided for appropriate use and calibration of these measurement procedures. Lastly, the document provides a summary of the objectives and status of the European project to develop higher order reference measurement procedures for free thyroid hormones.

2 Introduction[1-13]

When considering the concentration of free T4 (FT4) in serum or plasma, a clear distinction must be made between the *in vivo* situation and the *in vitro* condition that holds once a blood sample has been taken. In both, a dynamic equilibrium exists between free and bound T4 that depends on the concentration of the transport proteins as well as the affinity of their binding sites (which depends on the presence of competing ligands, pH, temperature, and other factors).

The *in vivo* relationship between FT4 and TT4 must be considered in terms of the free hormone hypothesis, which in its original and simplest form states that the biological effect (including disposal) of (thyroid) hormone is governed by its free, nonprotein-bound moiety, as can be measured *in vitro*. Especially this last statement is now considered a gross oversimplification, since it implies that everywhere in the body the same conditions prevail, as in the bulk of the circulation with unlimited availability of T4 and instantaneous equilibration. More sophisticated models postulate a complex interplay between flow, diffusion, and dissociation as means by which the hormone is made available to the target tissues.[13] The pivotal point here is the recognition that relative depletion of hormone may arise at the interface of cell structures within the microcirculation, when the hormone uptake rate by these cells is high. The magnitude of this depression inversely relates to the concentration of reversibly bound hormone. Nevertheless, these models should be considered as refinements or improvements of the free hormone hypothesis as long as hormone uptake by the cells is supposed to take place via the free form of the hormone, in contrast with models in which the hormone-transport protein complex enters the cell in its entirety or where hormone is transferred directly from the transport protein to a membrane transporter molecule.

The *in vivo* situation constitutes an open system into which the thyroid releases T4, while on the other hand, T4 is removed from the system by degradative processes. These, analogous to the biological effects, depend on the uptake rate of free hormone at the interface of the microcirculation and the tissues that harbor these processes. Notwithstanding the fact that local FT4 levels may differ from those in the bulk of the circulation, changes in serum T4 binding capacity initially lead to modified FT4 concentration(s) and therefore, modified uptake and ensuing T4 elimination. With T4 production remaining constant, the size of the circulating TT4 pool as well as FT4 levels change until a new steady state is attained in which the original serum FT4 (and thus T4 elimination rate) are restored. In this new steady state, TT4 has been adjusted corresponding to the change in serum T4 binding capacity.

In contrast, once a blood sample has been collected, it constitutes a closed system. Therefore, the TT4 concentration cannot change. Thus, while *in vivo,* the TT4 depends on the FT4 concentration, *in vitro,* the

opposite is true. Given a certain level of TT4, FT4 *in vitro* varies with the addition of binding sites and the presence of either inhibitors or promoters of binding.

The very first commercially available direct FT4 assay was marketed by Lepetit in 1977 and was based on sequestration to equilibrium of T4 from serum to porous polymerized dextran particles, followed by immunoassay of the T4 eluted. This procedure worked by virtue of the fact that the internal space of the dextran matrix was accessible to unbound T4 only. The necessary assay sensitivity could be attained, because the matrix concentrates T4 by a constant factor of about 40 in a dose-independent manner. In most if not all respects, this assay was theoretically equivalent to ED followed by T4 immunoassay in the dialysate. During the 1980s, there was great interest in immunoassay of FT4 based on assessment of antibody occupancy. If the T4-binding antibody constitutes a single order of noninteracting binding sites, the ambient FT4 concentration is directly proportional to the antibody occupancy ratio (i.e., the fraction of the total binding capacity that is actually occupied by the ligand) divided by the unoccupied binding capacity. This follows from the Law of Mass Action:

$$K_{diss} = [\text{Free ligand}] \cdot [\text{Free binding sites}]/[\text{Ligand-protein complex}]$$

Or:

$$[\text{Free ligand}] = K_{diss} \cdot [\text{Ligand-protein complex}]/[\text{Free binding sites}] =$$

$$= K_{diss} \cdot \{\text{Occupancy ratio}\}$$

In this relationship, the free ligand (FT4) concentration is the ambient concentration (i.e., the concentration in the equilibrium state that results from the effects of all factors involved). In real life assays, this may differ appreciably from the original concentration in the sample, as will be discussed.

Assessment of antibody occupancy *per se* is not required. There is even no need for actual attainment of equilibrium—though this may be desirable in view of assay stability—nor is it necessary to have a measurement signal that follows a simple relationship with antibody occupancy, as long as it is an unambiguous measure of occupancy; that is, samples with equal FT4 should give the same signal irrespective of their TT4 concentration. The unknown sample's signal is compared with the signal produced using a standard series of samples with known FT4. Therefore, these measurement procedures must be considered as "comparative."

A full account of all known assay principles can be found in Ekins' standard text.[4] There are three principal ways to obtain a measure of antibody occupancy:

1. *Two-step, back-titration:* In the first step, the serum sample is incubated with anti-T4 antibody, usually coupled to a solid support. The antibody will be occupied by a certain amount of T4, depending on the ambient FT4 concentration. The sample is washed away, and the solid-phase antibody (fractionally occupied by T4) is incubated with labeled T4. In this second step, binding of the label to the antibody exclusively depends on how much T4 has become bound during the first incubation and thereby exclusively depends on (ambient) FT4. Labeled T4 as a tracer may be replaced by a suitable analog of T4 that has a lower affinity for the antibody in order to attain better assay precision. Since the sample has been completely removed before addition of tracer, there are no special requirements of inertness toward serum components, such as a labeled analog has to meet in a one-step format.

2. *One-step, labeled analog:* Similar to the above principle, the occupancy of the antibody is reflected in the amount of label bound to the antibody. However, this principle differs from the previous one in that the sample is not separated from the antibody before addition of the label. Since binding of the label to the antibody must depend exclusively on the antibody-bound T4, the label must not interact with any sample constituent. Labeled analogs of T4 have been prepared for this purpose, but it has

been a subject of discussion whether the theoretical requirements are sufficiently met. In Appendix B, an estimate of the effect of analog binding to serum constituents is given.

3. *One-step, labeled antibody (unlabeled analog):* The difference between this measurement procedure and the labeled analog approach is in the location of the label. Essential to both approaches is the concentration of the analog-antibody complex being reflected by the label, which in principle may be located either on the analog or on the antibody. If located on the analog, the antibody-analog complex must be separable by immobilization of the antibody to particles or tube wall. If it is the antibody that is labeled, it is the analog that must be coupled to a solid support.

Theoretically, the labeled antibody approach must meet the same requirements as the labeled analog assay, as competition between antibody and serum proteins for the unlabeled analog will lead to lower binding of the labeled antibody to the immobilized analog. It seems, however, that immobilized analogs are more inert toward serum binding proteins than are labeled ones in the fluid phase.

Other commercial assays have also been described in which antibody occupancy was actually assessed or approximated. These were indirect assays, requiring TT4 assay and assessment of T4 tracer binding to antibody. Except for the labeled antibody assays, antibodies always have been immobilized, by coupling to tube walls, microdilution wells, microparticles, or microencapsulation. None of these designs has distinct theoretical advantages over the others.

The design of a measurement procedure should enable its user to produce accurate measurement results. However, in free hormone assays, inaccuracy has a special meaning. It is by no means solely due to improper calibration, but is also highly dependent on protein concentrations, binding capacities, and/or presence of inhibitors or promoters of binding. Inaccurate FT4 assays give different measurement signals in samples with equal FT4 but differing in TT4 concentration because of different T4 binding capacities. Two main causes for such inaccuracy can be identified: analog interacting with sample components and "sequestration" of T4 from the sample. Often, it is a combination of the two. With the exception of symmetric dialysis (SD), all FT4 assays sequester T4 from the sample. This may be by actual physical compartmentalization (ED or UF) or by the addition of a binding agent (usually specific antibody). Although the amount of T4 sequestered and the resulting decrease of ambient FT4 is a function of the FT4 concentration, the preexisting equilibrium is disturbed in a manner and extent that depends both on the binding characteristics of the sample as well as on the way in which the sequestration is achieved (see Appendix A). A differential effect on samples and standards leads to systematic inaccuracy or bias, and differential effects between patient samples lead to patient-dependent inaccuracy. As shown in Section 5.5.1, the dilution test is an appropriate test to detect the level of sequestration by assay reagents. It must be stressed that hormone binding to the antibody may not be the only form of sequestration. In many commercial assays, other binding proteins, mostly albumin, are added for various or no apparent reasons. One reason may be the suppression of tracer binding to serum constituents. An unfortunate side effect may be the increased sequestration of the hormone itself.

Under certain conditions, it is possible to adopt criteria for the degree of interaction of analog with sample constituents. This depends on the variability of these constituents normally encountered in the pathophysiological range. For example, it could be agreed upon what the maximal admissible effect, in terms of inaccuracy or imprecision, of severe hypoalbuminaemia might be on the assay result. The extent of interaction then can be derived by means of the theoretical model for this type of interference, given in Appendix B.

Critical judgment of the design of an FT4 assay should consist of identifying the nature of the disturbance of equilibrium during the analytical process and estimating the magnitude of its effects.

Disturbances of equilibrium can be subdivided into a few basic types that are discussed in Appendix A.

NOTE: Clinical application/strategies/interpretation of thyroid function tests and treatment guidelines are beyond the scope of this document.[11,12,14-23]

3 Standard Precautions

Because it is often impossible to know what might be infectious, all patient and laboratory specimens are treated as infectious and handled according to "standard precautions." Standard precautions are guidelines that combine the major features of "universal precautions and body substance isolation" practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (*Guideline for Isolation Precautions in Hospitals.* Infection Control and Hospital Epidemiology. CDC. 1996;17(1):53-80 and *MMWR* 1988;37:377-388). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of NCCLS document M29—*Protection of Laboratory Workers from Occupationally Acquired Infections*.

4 Definitions

Accuracy (of measurement) – Closeness of the agreement between the result of a measurement and a true value of the measurand (VIM93).[24]

Absolute FTH measurement procedures – Rely on standard hormone solutions in buffer as calibrant, whose concentrations have been established gravimetrically or by other similar analytical means; **NOTE:** Dialysis and UF measurement procedures (whether direct or indirect) typically fall into this category, since such calibrants are normally used either to measure the FTH concentration in the dialysate in the first case, or the total concentration in the sample in the second case.

Comparative FTH measurement procedures – Rely on use as calibrants of serum containing various total hormone concentrations, in which the corresponding FTH concentration is established by an absolute measurement procedure.

Direct FTH measurement procedures – Measurement procedures in which hormone is isolated from the protein-bound moiety prior to direct measurement of the amount sequestered (e.g., by immunoassay).

Free hormone fraction – A number between zero and unity indicating what proportion of hormone is in free, nonprotein-bound form; **NOTE:** Alternatively, the free fraction may be expressed as a percentage, which in the case of FT4 leads to more convenient figures.

Free thyroid hormone index (FTI) tests – All tests in which an estimate of total T4 (TT4) or total T3 (TT3) is required[25]; **NOTE:** This definition concerns two distinct categories of tests. First, those in which the total hormone measurement is combined either with a THBR test (e.g., T3-uptake) or a measurement of TBG. This approach leads to indices that correlate with, but cannot be considered as unambiguous estimates of the true FTH concentration. The second category comprises techniques like indirect ED, UF, SD, and gel equilibration that may yield valid estimates of the FTH fraction so that, after multiplication with the total concentration, a valid approximation of the true FTH concentration is obtained. It is recommended to reserve the term "FTI" for the first category of tests only, since it would be rather confusing when—at least in theory—potential reference measurement procedures would be classified under that name.

Immunoassay – A ligand-binding assay that uses a specific antigen or antibody capable of binding to the analyte.

Indirect FTH measurement procedures – Measurement procedures in which the FTH concentration is inferred from measurements of the total hormone concentration and the FTH fraction (e.g., by ED or SD) or a quantity reflecting the FTH fraction (e.g., fractional hormone uptake by a solid adsorbent such as solid phase antibody, ion-exchange resin, etc.).

International conventional reference measurement procedure – Measurement procedure yielding values that are not metrologically traceable to the SI, but which by international agreement are used as reference values for a defined quantity.[26]

Physical separation measurement procedures – Measurement procedures that employ a physical separation of the free and protein-bound hormone in the specimen; **NOTES:** a) Approaches typically used are based on separation across a membrane (e.g., in dialysis or UF), gel-filtration/bead analysis or gel adsorption, followed by direct quantitation of hormone in the free fraction by a sensitive measuring technique (e.g., immunoassay); b) Strictly speaking, immunoextraction also falls under this definition.

Precision of measurement – Closeness of agreement between independent results of measurements obtained under stipulated conditions (repeatability or reproducibility).[27]

Radioimmunoassay (RIA) – An analytical procedure in which an antibody and a radio-labeled analyte are used to measure the amount of analyte in a sample; **NOTE:** See also **Immunoassay**.

Reference measurement procedure – Thoroughly investigated measurement procedure shown to yield values having an uncertainty of measurement commensurate with its intended use, especially in assessing the trueness of other measurement procedures for the same quantity and in characterizing reference materials[28]; **NOTES:** a) Reference measurement procedures for SI-traceable measurands are based on matrix-independent measurement principles and are directly calibrated with gravimetrically prepared primary calibrator material[26,29]; b) With respect to measurands that are not (yet) SI-traceable, the term "international conventional reference measurement procedure" applies.

Serum binding capacity (sBC) – The reciprocal of the FTH fraction, which is proportional to the number of free hormone binding sites and binding affinity.

Single test ("direct") ligand assays – Rely on observations of the reactions between "binding pairs," one of which may be, e.g., an antibody or binding protein; **NOTE:** These measurement procedures may consist of three different formats: "two-step," "one-step labeled hormone-analog," and "one-step labeled antibody" (see Section 2, Introduction).

Thyroid hormone binding ratio (THBR) tests – Techniques to mathematically adjust total hormone concentrations for binding protein effects; **NOTES:** a) The terminology "THBR" replaces outdated terms such as "T3-uptake" or "T-uptake"; b) THBR tests estimate the concentration of thyroid hormone binding proteins in the serum sample; c) According to the Law of Mass Action, a THBR assay should measure the total "thyroid hormone binding capacity," in which the concentration of all thyroid hormone binding proteins influences the result. In spite of this, some THBR assays are designed to measure primarily the dominant thyroid hormone binding protein, namely thyroxine binding globulin (TBG).

Traceability – Property of the result of a measurement or the value of a standard whereby it can be related to stated references, usually national or international standards, through an unbroken chain of comparisons all having stated uncertainties.[24]

Trueness (of measurement) – Closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.[27]

Uncertainty of measurement – Parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand.[24]

Glossary of Abbreviations

ED	equilibrium dialysis
FDH	familial dysalbuminemic hyperthyroxinemia
FFA/NEFA	free fatty acid/nonesterified fatty acid
FTH	free thyroid hormone
FTI	free thyroid hormone index
FT3	free T3
FT4	free T4
HAMA	human antimouse antibodies
HEPES	N-(2-hydroxyethyl) piperazine-N´-(ethanesulfonic acid)
ID-MS	isotope dilution-mass spectrometry
NTI	nonthyroidal illness
QC	quality control
(R)IA	(radio)immunoassay
rT3	reverse T3, O-[4-hydroxy-3,5-diiodophenyl]-3-iodo-L-tyrosine
sBC	serum binding capacity
SD	symmetric dialysis
SI	Système International d'Unités
T3	triiodo-L-thyronine, O-[4-hydroxy-3-iodophenyl]-3,5-diodo-L-tyrosine
T4	L-thyroxine, 3-[4-(4-hydroxy-3,5-diiodophenoxy-3,5-diiodophenyl]-L-alanine
TBG	thyroxine binding globulin
THBR	thyroid hormone binding ratio
TRIAC	3-3´,5-triiodothyroacetic acid
TTR/TBPA	transthyretin/thyroxine binding prealbumin
TT3	total T3
TT4	total T4
UF	ultrafiltration

5 "Current" Practice for Assessment of Analytical Validity, Accuracy/Trueness

5.1 Analytical Performance of Free Thyroid Hormone Measurement Procedures

As indicated above, the analytical performance of an FTH measurement procedure should be such that it enables its user to produce accurate measurement results in different patient categories. Note that the concept "accuracy of measurement" is related to "trueness of measurement" and "precision of measurement" (see Section 4, Definitions). Whether a measurement procedure has this capability or not depends on its intrinsic quality, on the correctness of calibration and on the robustness of the measurement procedure to variations in the individual sample matrix.

Assessment of the intrinsic quality of a measurement procedure implies evaluation of common analytical characteristics such as imprecision, recovery, interferences, etc. (See Sections 5.2 and 5.3.)

Correctness of calibration is a metrological issue. For well-defined quantities such as T4 and FT4, for which expression of measurement results in the International System of Units (SI units) applies, it depends on the metrological traceability of the values of the calibrators through an unbroken chain of comparisons having stated uncertainties. The object of using SI-traceable calibration of routine measurement procedures is to produce accuracy/trueness of measurement (i.e., results of measurement that are as close as required to that obtained with an SI-traceable reference measurement procedure). Thus, validation of the traceability of a routine measurement procedure's calibration requires split-sample

measurements with an SI-traceable reference measurement procedure (see Section 5.6). These measurements should be done on a panel of patient specimens with FTH concentrations as evenly distributed as practicable over the working range of the assessed measurement procedure. However, SI-traceability of calibration with trueness of measurements in average (for the panel of patient samples) is only one issue of analytical validity. Accuracy at the level of the individual sample is equally important, in particular because serum/plasma FTH concentrations are governed by protein binding constants and equilibria between free and total hormone moieties. Therefore, investigation of the robustness of the FTH measurement procedure in the face of variations in the total hormone concentration, protein binding capacities/affinities, and the possible presence in test samples of endogenous or exogenous TH binding competitors (such as free fatty acids [FFAs] or drugs) should also be part of the assessment of the analytical validity. This assessment should be done by challenging the FTH measurement procedure with, e.g., dilution of serum, addition to the serum of thyroid hormone binders, etc. (See Section 5.5.) Additionally, it should comprise correlation studies of measurement results with those obtained by a measurement procedure, which is essentially independent of the previously described variations in the individual sample (not necessarily an SI-traceable reference measurement procedure). This correlation study also should be done with a panel of patient specimens representing a broad range of clinical pathologies and serum thyroid hormone binding capacities (see Sections 7.2 to 7.4).

5.2 Analytical Validation of Free Thyroid Hormones

Some aspects of analytical performance of FTH measurement procedures can be assessed using current NCCLS guidelines. Other performance aspects are specific to FTH measurement procedures and are, therefore, not incorporated in current NCCLS documents. These will be discussed in detail in this document.

5.2.1 Imprecision

It has been proposed that analytical goals of imprecision and bias of any assay could be derived from the components of biological variation, namely, within-subject (CV_I) and between-subject (CV_G) variation.[30] For imprecision and bias, the desirable performance of an assay (CV_A) could be defined as $CV_A < 0.5CV_I$ and $B_A < 0.25(CV_I^2 + CV_G^2)^{1/2}$, respectively. Published CV_I and CV_G for FT4 in euthyroid subjects are 9.5% and 12.1%, respectively (the corresponding values for FT3 are 7.9% and 12.1%).[31] Thus, the calculated desirable analytical goals for imprecision and bias for euthyroid FT4 levels are 4.8% and 3%, respectively (the corresponding values for FT3 are 4% and 3%).

The within-day, within-calibration, and within-laboratory imprecision should be evaluated using the current edition of NCCLS document EP5—*Evaluation of Precision Performance of Quantitative Measurement Methods*. These studies should be carried out using QC samples that have FTH concentrations representative of hypothyroid, euthyroid, and hyperthyroid populations. It is also of paramount importance that the QC materials used by manufacturers (and users) to monitor analytical performance mimic the performance obtained with patient sera.

Recommendation: Manufacturers should strive to meet these goals. Data on within-run, between-run, and between-lot imprecision and lot-to-lot variability in bias at clinically important doses should be made available to users.

5.2.2 Linearity

The linearity (or test of parallelism to the standard curve) protocol described in the current edition of NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach* examines the relationship between the expected concentration of an analyte to the concentration actually measured by the assay. The test involves dilution of a sample containing a high hormone concentration with a sample which is devoid of the hormone (or contains a very small amount of

the hormone). The calculated concentration of hormone in the diluted samples (at least five dilutions are required to be performed) are then compared (by linear regression) to the concentration measured by the assay. Data can then be used to define the highest and lowest limits of the linearity claim. This evaluation protocol is not applicable to FTH measurement procedures. This is because the FTH concentration of the diluted pools cannot be readily predicted, as it will depend on the sBC of the high and low (or zero) concentration pools. This is exemplified by the data depicted in Figure 1, which shows the FT4 concentration obtained, by simulation, when a high FT4 serum is diluted (2-fold to 16-fold) with three different "zero" FT4 sera that have different thyroid hormone binding capacities. The x-axis depicts the predicted FT4 concentration, based on the known FT4 concentration in the "high" sample divided by the relevant dilution factor. The measured FT4 values of the diluted samples are depicted on the y-axis. Increased FT4 measured values would be observed for serum 1, which has a lower thyroid hormone binding capacity compared to the high FT4 serum. A decrease in the measured FT4 values would be observed for serum 3, which has a higher binding capacity.

One possible way to overcome the inability to predict the FT4 concentration is to actually measure the serum dilution pools in a reference measurement procedure, thus allowing the regression analysis to be performed per NCCLS document EP6—*Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*.

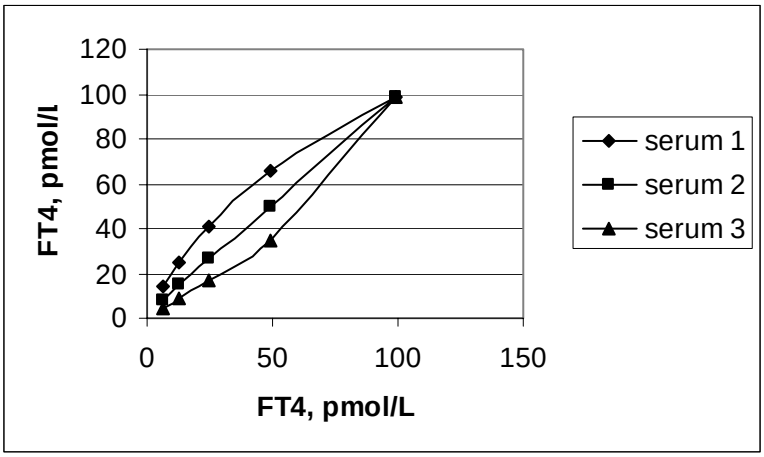

Figure 1. FT4 Concentration Obtained, by Simulation, When a High FT4 Serum is Diluted (2-fold to 16-fold) with Three Different "Zero" FT4 Sera That Have Different Thyroid Hormone Binding Capacities. The x-axis depicts the predicted FT4 concentration, based on the known FT4 concentration in the "high" sample divided by the relevant dilution factor. The measured FT4 values of the diluted samples are depicted on the y-axis.

5.2.3 Recovery

Classically, the calibration of an assay is assessed by estimating the % recovery of known concentrations of the pure analyte spiked into patients' sera. This test is not appropriate for FTH, because the concentration of FT3 or FT4 will be dependent on both the concentration of the "pure" analyte (T3 or T4) and the sBC (concentration and affinity of the T3 and T4 binding proteins) of the patients' sera. The only way that "recovery" of an FTH assay can be assessed is by comparing the magnitude of the FTH change incurred, following addition of the pure analyte into patients' sera, in the test method and that obtained by the reference method.

5.2.4 Serum/Plasma Differences and Effect of Storage Conditions

The suitability of serum and plasma (EDTA, heparin) obtained in different collection devices needs to be investigated by the manufacturer and appropriately claimed in the product literature. Also, the ideal

storage conditions (storage time and temperature, and the effect of freeze/thaw) should be included in the product literature.

Recommendation: It is the manufacturer's responsibility to define the type of specimen and optimal storage conditions that can be used in the assays.

5.3 Interference Testing

Interferents are defined as substances that cause an artifactual increase/decrease in the FTH concentration.

NOTE: *In vivo* drug interactions (such as changes in hormone concentration due to drug-induced physiological responses) should not be misinterpreted as interferents and are, therefore, dealt with in Section 5.3.2.

5.3.1 *In Vitro* Interferents

The effect of *in vitro* interferents (such as hemoglobin, bilirubin, lipids, and nonesterified fatty acids (NEFAs)[a] can be assessed following the protocols specified in EP7—*Interference Testing in Clinical Chemistry*. Other potential "assay-specific" interferents should be tested (e.g., high levels of biotin could affect the performance of assays that use streptavidin, or the presence of fluorescent substances in sera could affect assays using fluorescence as assay signal).

5.3.2 *In Vivo* Drug Interactions (Physiological Response)

Drugs known to displace thyroid hormones from their binding proteins (and thus, to cause an increase in the concentration of FTH) include the following: acetylsalicylic acid, amiodarone, diclofenac, diphenylhydantoin, furosemide, mefenamic acid, phenylbutazone, salicylate, and thyroid hormone metabolites. The effect of these substances on FTH concentration can be assessed following the experimental protocols described in NCCLS document EP7—*Interference Testing in Clinical Chemistry*. Briefly, this involves spiking a serum sample (preferably from a euthyroid patient) with a solution containing the drug or solvent and then estimating the FTH concentration. The amount of solvent spiked should be kept to an absolute minimum, and the independent effect of the solvent should be measured. However, it is important to recognize the limitations of this approach in predicting the FTH response of a patient to an administered drug. The main reason is in the fact that FT4 is regulated so that the organism tends to keep the FTH at a constant level in spite of modified binding (see Section 2, Introduction). Also, the magnitude of the FT3/FT4 increase (this occurs because these drugs can bind to the thyroid hormone binding proteins and displace FTH) will not only depend on the dose of the drug but also on the concentration and affinities (i.e., the binding capacity) of the serum binding proteins and of the TT4 concentration of the spiked sample. For example, the increase in FT3/FT4 seen when a drug is added to a low binding capacity serum will be much greater than that seen when the drug is added to a high binding capacity serum. Second, it should be recognized that the increase in FT3/FT4 occurring *in vivo* when drugs are administered to a patient would also depend on the time of sampling and the influence of homeostatic mechanisms. For example, a drug-induced FT4 elevation will cause a reduction in TSH, which in turn will reduce the TT4 concentration (and "normalize" the FT4 concentration). The FTH validity of assays not exhibiting an increase in the FTH concentration should be questioned. For example, this test, among others, was instrumental in identifying the deficiencies of a labeled analog FT4 measurement procedure.[32]

[a] NEFAs are normally generated *in vitro* through the actions of lipoprotein lipases, and because they are able to bind to the thyroid-binding proteins, they cause a "false" elevation in the FTH concentration. This is usually not a major problem, as the concentration of NEFAs in serum is normally too low to have any significant effects. However, a significant *in vitro* generation of NEFAs can occur when patients have been given heparin. *In vivo* administration of heparin stimulates the production of lipoprotein lipases, which act to release NEFAs.

5.3.3 Interference by Antibodies

Robustness of the assays to samples containing other potential interferents such as human antimouse antibodies (HAMAs), heterophilic antibodies, and rheumatoid factors should be assessed and every effort made to reduce/eliminate the interference. Classically, such interferences are reduced by the addition to the assay reagents of animal sera and globulin fractions (IgG and IgM). For free hormone assays, only globulin fractions, not animal sera, should be used. This is because animal sera contain thyroid hormone binding proteins, and their presence in the assay reagents could compromise the free hormone validity of the assay (see Section 5.5.2). It is difficult to estimate the prevalence of interference, as it will be dependent on the immunoassay used (and the amount of animal IgG added to the assay reagents). In the absence of these additives, the incidence could be as high as 30% of the population.[33] It is important to note that it is impossible to ensure complete robustness of the assay to these types of interferents, and this fact needs to be communicated to the users. Inconsistencies between thyroid function tests may be a hint of the presence of such interferences. Manufacturers should recommend troubleshooting protocols, which customers can use to investigate the presence of such interferents. T3 and T4 autoantibodies constitute a special case since they are in effect unusual thyroid hormone serum binding proteins, albeit possibly possessing very different on and off rates from the thyroid hormone binding proteins normally present in serum. It is not known what effect they have on hormone transport to target tissues, although most of the available literature[11] suggests that both the FTH and TSH concentrations in these patients are within the euthyroid range. Some FTH assay formats (e.g., labeled analog and to a lesser extent labeled antibody measurement procedures) are prone to interference from such autoantibodies.

5.3.4 Abnormal Binding Proteins

The performance of FTH assays should also be challenged in situations where the binding protein concentrations, and thus the concentration of TT4 or TT3, are abnormal. This requires a population of specimens from patients having congenitally increased, as well as decreased, thyroid hormone binding capacities that are within the euthyroid range (see Section 7).

Recommendations

- Physicians and clinical biochemists should be informed regarding the effects of medications on FTH tests.
- Manufacturers should provide information on the expected assay performance of specimens from patients with abnormal levels of serum binding proteins (and thus, abnormal concentration of TT4 or TT3).
- Physicians and clinical biochemists should be made aware that despite all efforts to minimize interferences by HAMAs and heterophilic antibodies, it is impossible for the manufacturer to fully ensure the lack of interference. A “limitation” statement should be included in the product literature. Manufacturers should devise troubleshooting protocols, which can be followed by users when investigating the presence of such interferents.

5.3.5 Cross-Reactivity

It is important for manufacturers to determine the binding characteristics (e.g., degree of cross-reactivity of chemically related substances) for the antibodies used in FTH assays. However, it is not possible to estimate the cross-reactivity of FTH assays using “classical” protocols. This is because all potential cross-reacting substances can displace thyroid hormone from their binding proteins (and thus, elevate the FTH concentration). Therefore, it is almost impossible to distinguish “antibody cross-reactivity” from an effect on the T4 binding proteins. It is essential that cross-reactivity assessment be performed in a buffer system devoid of any thyroid hormone binding proteins. This can be done by preparing the T3/T4 “standards”

and the potential interferents in buffer (e.g., HEPES (N-[2-hydroxyethyl] piperazine-N′-[ethanesulfonic acid], or phosphate buffer containing 0.1% gelatin). Following estimation of the T3/T4 immunoreactivity of the potential interferents, one can calculate their % cross-reactivity (defined as 100 x (immunoreactivity)/(actual concentration)). Substances that should be assessed include triiodo-L-thyronine, L-thyroxine T4, 3,3',5-triiodo-L-thyroacetic acid (TRIAC), 3,3',5'-triiodo-L-thyronine (rT3), 3,5-diiodothyronine, 3,5-diodo-L-tyrosine (DIT), D-thyroxine, and 3-iodo-L-tyrosine (MIT).

Recommendation: Manufacturers should provide specificity data (degree of cross-reactivity by chemically related substances) and details of the measurement procedure used for this assessment.

5.4 Assay Dynamic Range

It is recommended that the dynamic range (defined as the concentration range which meets imprecision goals) for measuring FT3 and FT4 assays should be approximately 25% of the lower euthyroid limit to 400% of the upper limit of the euthyroid range for appropriate clinical utility.

5.5 Recommended Protocols for Assessing the Validity of Free Thyroid Hormone Assays

Using equations derived from the Law of Mass Action, a number of experiments can be devised that will challenge the validity of FTH assays. These are described below.

5.5.1 Effect of Serum Dilution

Serum dilution is indicative of an assay's validity for testing various patient sera. It can be theoretically predicted that dilution of euthyroid sera with an inert buffer will cause a minute change in the FTH concentrations. For example, serial dilution (up to an eightfold dilution) of a euthyroid serum with 10 mmol/l HEPES pH 7.4 will be expected to reduce the FT4 concentration by no more than 1% (FT3 will be expected to show a <5% decrease). Assays exhibiting dilution-dependent biases are expected to yield inappropriately low results in patients whose sera contain low concentrations (or affinities) of T4 binding proteins. This test is indicative of the assay's validity, and whether or not it is likely to give misleading results in other patient categories. Figure 2 shows the serum dilution profiles expected when the binding capacities of the antibody are 0.0%, 0.2%, 0.5%, 1%, 5%, and 15% of the total binding capacity in the immunoassay tube. In these situations, the antibody will bind (or have sequestered) 0.2 to 15% of the total serum T4. The serum used for these simulations was a euthyroid serum containing midnormal concentrations of TT4 and thyroid hormone binding proteins; the immunoassay protocol used 25 µL sample in a total reaction volume of 125 µL (with the only T4 binder in the reagents being the antibody). The sample was serially diluted to 32-fold, which equates to a final serum dilution of 1/160.

These simulation results suggest that the FT4 concentration is robust to serum dilution, as long as the antibody binding capacity is kept to a minimum (e.g., T4 sequestration should be less than 0.5% of the TT4 concentration). At higher binding capacities, the FT4 concentrations decrease in parallel to the dilution factor; the dilution-induced reduction in FT4 becomes greater as the binding capacities increase. This simulation also highlights the fact that different immunoassays can be made (by appropriate calibration) to produce similar results in patients having normal binding protein and TT4 concentrations, but significant methodological differences will be evident when low binding capacity sera (as simulated by the serum dilution) are analyzed. The magnitude of the bias (negative direction) will be greater in assays whose reagents sequester higher amounts of TT4. Conversely, a positive bias will result in sera with high binding capacity, as more T4 is sequestered from the standard serum than from the high binding capacity serum. Likewise, the effect will be larger, the more T4 is sequestered. The mathematical description of the effect of serum dilution can be found in Appendix A.

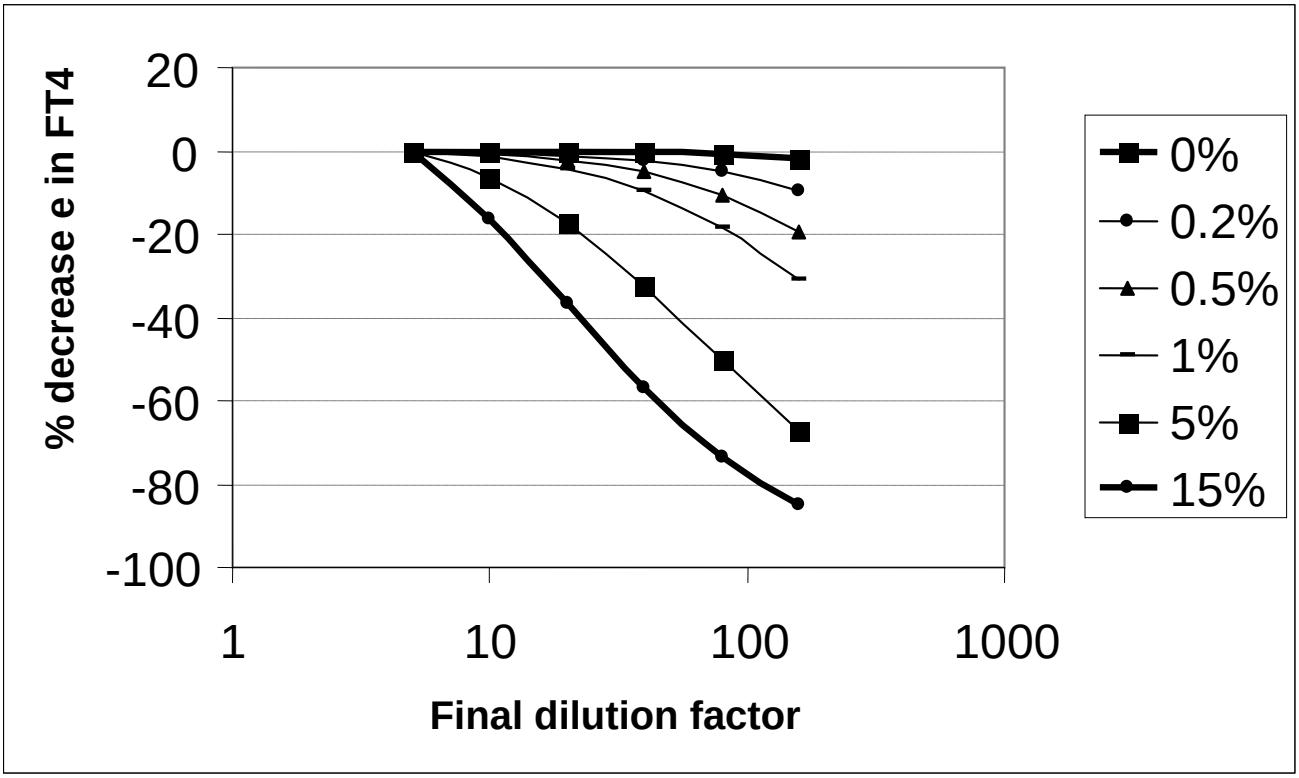

Figure 2. Simulated Serum Dilution Profiles When the Binding Capacity of the Antibody is Varied From 0 (i.e., no antibody added) to Situations When It is 0.2%, 0.5%, 1%, 5%, and 15% of the Total Binding Capacity in the Immunoassay Tube. (Used with permission. Christofides ND. Free analyte immunoassay. In: Wild D, ed. *The Immunoassay Handbook*. London: Nature Publishing Group; 2001:61-77.)

5.5.2 Addition to Serum of a Thyroid Hormone Binder

It can readily be shown that the FTH concentration is equal to the concentration of protein-bound hormone divided by sBC. Thus, it can be predicted that exogenous addition of a thyroid hormone binder (such as albumin or TBG) will cause a decrease in the FTH concentration (by increasing the denominator, the sBC term, in the equation). An apparent increase (or no change) in the FT4 concentration following addition of thyroid hormone binders should question the free hormone validity of the assay. The results obtained in the test assay should be compared to those obtained in the reference method.

Protocol: Increasing amounts of TBG (0 to 40 mg/L) and human serum albumin (0 to 80 g/L) are added to a euthyroid serum and the samples assessed in the test and reference FT4 and FT3 assays.

5.5.3 Addition to Serum of Substances that Cause Displacement of Thyroid Hormones from Their Binding Proteins

Agents that are capable of displacing thyroid hormones from their binding proteins will be expected to cause a dose-dependent increase in FTH concentration when added to patient sera. Agents that cause displacement of thyroid hormones from their binding proteins include fatty acids and drugs that have structural similarities to thyroid hormones (described under the "*in vivo* drug interaction" section above). The protocol used for these experiments is described in NCCLS document EP7—*Interference Testing in Clinical Chemistry*. It should be recognized that both the dose of the drug and the binding capacity of the spiked serum will influence the magnitude of the FT3/FT4 elevation. The effect of these drug interactions should be compared to those obtained in the reference measurement procedure (when this is available). The validity of FTH assays that do not show the same increase in FT3/FT4 as the reference measurement

procedure should be questioned. The absence of an increase in the FT4/FT3 or a fall in their concentration following addition of substances known to cause a displacement of thyroid hormone is consistent with analog binding to thyroid hormone binding proteins. In the case of *in vitro* addition of FFA (e.g., oleate), the presence of albumin in the assay buffer may cause the absence of an increase in FT4/FT3, since this protein very avidly binds FFA.

Recommendation: It is the manufacturers' responsibility to provide users (the laboratory and the physician) with detailed information of the assay's free hormone validity using one of these approaches.

5.6 Validation by Comparison with a Reference Measurement Procedure

The validation experiments described in Sections 5.1 to 5.5 will provide useful information on the analytical performance of a routine measurement procedure. However, ideally, clinical specimen results from that measurement procedure should also be compared to those from a reference measurement procedure to document traceability. Unfortunately, there is currently no officially endorsed reference measurement procedure. Three candidate procedures are described in the following sections: direct ED, UF[4], or indirect SD calibrated by ED or UF.[34]

5.6.1 Direct Equilibrium Dialysis as Potential Reference Measurement Procedure[35-39]

Direct ED uses a dialysis cell that consists of two compartments that are separated by a semipermeable membrane, characterized by its molecular weight cutoff (the cell should be held at 37 °C). One compartment (referred to as dialysand) is filled with the serum sample, brought to pH 7.4 by the addition of a small amount of buffer. The other compartment contains solely the buffer (= the dialysate). With the exception of proteins (for example, the FTH binders) and other high molecular weight serum components, substances can diffuse freely across the membrane. Equilibration between the FTH in the serum compartment and the hormone in the dialysate is reached after an appropriate incubation time (typically 16 to 24 hours, although in some dialysis cells, relying (for example) on a high dialysand/dialysate volume-ratio, equilibrium may be reached within six hours). Usually, a highly sensitive immunoassay is used to determine the thyroid hormone concentration in the dialysate. A direct ED measurement procedure (based on the literature of Nelson and Tomei[38]) is commercially available; however, manufacturers of FTH assays may wish to develop their own procedures.

Whether the concentration of FTH measured by direct ED is equal to the concentration in the original serum sample depends on a number of factors:

- Chemical composition and pH both of the buffer in the dialysate and of the buffer used to control the serum pH, as they profoundly affect the affinity of the binding proteins.

  NOTE: If specific ions in the serum that affect T4 binding affinity are absent in the dialysate buffer, their concentration will be reduced at dialysis equilibrium with respect to their original concentration. More specifically, the absence of a dialyzable inhibitor of T4 binding, such as the chloride ion from the dialysis buffer, results in a lower overall chloride concentration, therefore, less inhibition of T4 binding and hence, lower FT4[39] at equilibrium.

- The dilution factor of the serum prior to dialysis and the dialysand/dialysate volume ratio. It should be recognized that the technique of ED implicitly involves serum dilution due to the fact that a certain minimum volume of buffer is required at the opposite side of the membrane in order to make the measurements technically feasible.

  NOTE: The diluent volume comprises the total of the amounts of diluent in the dialysand and dialysate. With a certain total diluent volume, the FTH concentration is independent of whether the

serum has been prediluted or not, provided no nondialyzable inhibitors of T4 binding are present (see Appendix A). This is of importance when comparing ED results with those of UF and SD.

- The temperature of dialysis (since the affinity of proteins is temperature dependent).

  NOTE: This applies for all measurement procedures.

- Generation in the dialysand of dialyzable or nondialyzable binding inhibitors (e.g., FFA by conversion of triglycerides), also dialyzable inhibitors.

  NOTE: Checking results using different predilution factors and/or changing dialysate volumes offers a means of establishing whether binding competitors are present in sufficient amounts to affect assay results.

- The nature of the semipermeable membrane and cell surfaces (as they affect permeability/protein leakage/nonspecific binding).

- The analytical procedure for measurement of the thyroid hormone in the dialysate and its calibration (as it affects the accuracy of the thyroid hormone determination in the dialysate).

Because of the importance of the above factors with respect to the balance of the free and bound hormone moieties in the original serum matrix, it is recommended to perform direct ED as described below.

Recommendations

- The dialysate buffer should have a biochemical composition that imitates the ionic environment of the serum as closely as possible. Since adjusting and maintaining pH 7.4 in a more physiological way (i.e., Krebs-Ringer bicarbonate buffer in O_2/CO_2 atmosphere) during dialysis is technically not feasible, the use of HEPES is a good compromise (e.g., the buffers proposed by Ellis and Ekins or Nelson and Weiss).[35, 40]

- The serum sample should be buffered at pH 7.4 by adding a minimum volume of HEPES buffer (e.g., 50 µL of 1 mol/L HEPES buffer per mL of serum).[41]

- The sample should not be diluted before dialysis, and the dilution factor inherent to dialysis should not exceed 1:2 (i.e., by using equal volumes of serum and dialysate buffer[40]) (contrary to the procedure described in Nelson and Tomei,[38] implying a serum dilution (by the dialysate) of 1:5).

  NOTE: Neither Nelson and Weiss[40] nor Nelson and Tomei[38] took serum dilution by dialysate into account in their experiments.

- The temperature during dialysis should be kept at 37 °C (±0.5 °C, using a calibrated thermometer) and kept constant (within a ±1 °C interval).

- The semipermeable membrane should preferably have a nominal cutoff of 10 kDa (a low cutoff value is desirable, since separation efficiency is influenced by pore size distribution and the presence of pores larger than the average. The latter increases the risk of protein leakage with membranes of higher nominal cutoff).

- The membrane should preferably consist of regenerated cellulose[41,42] (membranes made of materials such as polysulfone and polyethersulfone show unacceptably high adsorption).

Recommendations (Continued)

- Adsorption to the dialysis cell surface and membrane should be assessed with T4 tracer and avoided by adequate pretreatment.[38,43]

- To prevent significant adsorption losses of T4 in the dialysate, collection should be done in tubes containing, e.g., 0.2 % albumin solution.

  NOTE: Ellis and Ekins used gelatin.[35]

  The albumin should be assessed for its intrinsic thyroid hormone content (to cause less than 1% error).

- Determination of the thyroid hormone in the dialysate should preferably be done with an immunoassay which has been calibrated via method comparison on patient samples with a reference measurement procedure (that can, contrarily to an immunoassay, be directly calibrated with gravimetrically prepared primary calibrator material and give true measurement results).

5.6.2 Direct Ultrafiltration as a Potential Reference Measurement Procedure[44-48]

UF is another technique used to separate substances according to molecular weight and size. In UF, serum is forced via gas pressurization, vacuum, or centrifugation through a membrane characterized by its cutoff value.

NOTE: The compartment containing the substances that cannot permeate the membrane is referred to as retentate; the other compartment contains the ultrafiltrate.

After molecular filtration, the thyroid hormone concentration in the ultrafiltrate is measured by immunoassay (direct UF). UF is the only technique that can separate the FTH from the protein-bound hormone without dilution of serum. It also typically permits separation of solutes in a shorter time than is possible using dialysis.

However, as with ED, the assumption that the FTH measured by UF is equal to the concentration in the original serum sample depends on the following factors:

- the pH of the sample and the temperature during the membrane filtration process (as they affect the affinity of the thyroid hormone binding proteins).

- the nature of the membrane and the UF hardware (construction, surfaces, etc.) as they affect permeability/protein leakage/nonspecific binding.

  NOTE: In UF, the risk for protein leakage is greater, because the filtration process is driven by mechanical forces.

- the degree of change in concentration of nonfiltrable T4 binding competitors present in the serum compartment, e.g., FFA (as this progressively increases the FTH concentration appearing in the ultrafiltrate).

  NOTE: This factor is particularly critical, since concentration of this kind of component in the retentate is inherent to the UF process.

- the Donnan effect (that is, unequal charge distribution over the protein and free ligand compartments), leading to unequal distribution of FTH in the ultrafiltrate and retentate and dependent on the UF fraction.

- the analytical procedure for measurement of the thyroid hormone in the ultrafiltrate and its calibration (as it affects the accuracy of the thyroid hormone determination in the ultrafiltrate).

To prevent or minimize the above intrinsic limiting factors, valid direct UF, like ED, requires that adequate measures, as recommended below, are taken.

Recommendations

- The serum sample should be buffered at pH 7.4 by adding a minimum volume of HEPES buffer (cf. ED).

- The serum sample should not be diluted prior to UF or, to obtain results in agreement with direct ED, dilution should not exceed a 1:2 ratio.

- After filling the serum compartment, the UF device should be capped or covered with self-sealing, moldable, flexible film to prevent concentration of serum by evaporation.

- The filled UF device should be brought to 37 °C before starting the molecular filtration process.

- During the entire UF process, the temperature should be kept at 37 °C (cf. ED).

- The semipermeable membrane should have a nominal molecular weight cutoff of 10 kDa (cf. ED) or 3 kDa (investigations currently performed in the EU Project).

- The membrane should preferably consist of regenerated cellulose (cf. ED).

- Adsorption to the membrane and the surfaces of the retentate compartment should be assessed with FT4 tracer (cf. ED).

- To prevent significant adsorption losses, a 0.2% albumin solution should be added to the ultrafiltrate compartment before the UF is started (cf. ED). The albumin should be assessed for its intrinsic thyroid hormone content.

- Protein leakage should be assessed with a test system sensitivity of 0.002% (e.g., expressed as albumin concentration of <1667 pmol/L).[42]

- In order to minimize biochemical changes during UF due to change in concentration and the Donnan effect, the UF fraction should be kept <30%.[49]

- Determination of the thyroid hormone in the dialysate should preferably be done with an immunoassay which has been calibrated via method comparison on patient samples with a reference measurement procedure (that can, contrarily to an immunoassay, be directly calibrated with gravimetrically prepared primary calibrator material and give true measurement results).

5.6.3 Symmetric Dialysis Calibrated by Equilibrium Dialysis or Ultrafiltration[34,50]

SD and ED share common hardware. However, SD contains identical solutions (sera) at both sides of the semipermeable membrane and is based on indirect measurement of the fraction of FTH. The technique

starts by adding radiolabeled hormone on one side of the membrane. This tracer rapidly equilibrates with endogenous hormone and diffuses to the other compartment, due to a concentration gradient of labeled hormone across the membrane. In theory, this process may continue to final equilibrium, so that tracer concentration becomes equal on either side of the membrane. However, to be able to deduce the FTH fraction, terminating the procedure before equilibrium is reached is a fundamental requirement of the approach. It is the rate at which the label migrates to the other compartment that is unambiguously related to the fraction of FTH. A simple, well-defined mathematical relationship permits calculation of the dialysis rate from tracer distribution at a single point in time. The dialysis rate depends, for a certain type and surface area of the membrane and sample volume, on the FTH fraction only. This is true when the dialysis rate is fully controlled by diffusion through the membrane. Potentially, there are two candidate processes that might be rate-controlling: dissociation of the ligand-binding protein complex and diffusion of ligand toward and from the membrane/solution interface through the hypothetical unstirred layer (film) at the membrane-solution interface. Comparison of dialysis rates with (low) estimates of dissociation rate constants of the T4-TBG complex virtually rules out the former. According to existing models for diffusion at the interface, film diffusion rate control is more likely to occur with relatively high free ligand fractions (i.e., not in undiluted serum) combined with insufficient agitation of the solutions lining the membrane. Experiments that were performed to create conditions that are favorable to film diffusion rate control (by keeping cells completely unstirred for 24 hours) made it highly unlikely that in normally agitated cells such a mechanism is relevant.

From the above described technique, it should be recognized that SD is free from some limiting factors inherent to direct ED and UF. There is no separation of bound and free hormone, since the only process taking place is redistribution of labeled hormone. Thus, when applied to undiluted serum, disturbance of equilibrium is negligible (although a minimal disturbance due to pH adjustment of the serum to 7.4 and addition of the tracer cannot be avoided). In addition, it works with identical solutions at both sides of the membrane; thus, their composition is constant during the entire dialysis process. Furthermore, the presence of binding proteins on both sides of the membrane prevents adsorption of ligand to membrane and cell walls. However, some problems can be encountered in SD, including sample deterioration (as in ED) and tracer impurity. The main intrinsic disadvantage, however, is that SD requires calibration. The determination of the FTH fraction from the measurement of the dialysis rate depends on calibration using a solution whose true FTH fraction has been assessed with direct ED or UF. Finally, the free fraction has to be converted into FTH concentration by multiplying by the total hormone concentration.

In summary, factors of importance with respect to the validity of SD are:

- purity of the radiolabeled hormone tracer;
- adequate mixing;
- calibration;
- the total thyroid hormone concentration; and
- dialysis time in view of the generation in the serum of nondialyzable binding inhibitors (cf. ED).

Therefore, valid performance of SD requires strict adherence to specific measures.

Recommendations

- Tracer should be purified to contain impurities below 0.15% iodide.
- Use of a calibrator consisting of a thyroid hormone binding protein solution (without binding inhibitors) and with a thyroid hormone content at a level that allows optimal determination with a reference measurement procedure (e.g., a 2% albumin solution in HEPES buffer containing 5 µmol/L thyroid hormone).
- Construction of a dialysis rate vs. FTH fraction calibration curve in order to determine the linear range of the relation. Subsequent measurements should be carried out in this linear range.
- Determination of total thyroid hormone concentration with a reference measurement procedure.

6 Proposal for a Future Reference Measurement System

Although accuracy/trueness-based standardization of routine measurement procedures goes back to pioneering work done in the 70s,[35, 51-54] its implementation in practice by the *in vitro* diagnostic industry was realized only recently, mainly driven by recent legislative regulations in Europe.[55]

According to the IVD Directive, EC declaration of conformity requires, among other things, that the technical documentation of an *in vitro* diagnostic medical device includes performance evaluation data supported by a reference measurement system (when available). In order to provide industry with tools to comply with the requirements by the IVD Directive and its accompanying standard,[26] a project was started in Europe to support the development of such a reference measurement system, comprising a reference measurement procedure and certified reference materials, for *in vitro* thyroid diagnostic testing.[56] With respect to FTH measurements, the main objective of the project is to investigate whether ED or UF can be elevated to the status of a reference measurement procedure. Theoretically, the basic requirements of a reference measurement procedure are fulfilled by direct ED and UF, provided (1) the recommendations for their performance given in Sections 5.6.1 and 5.6.2 are strictly followed and (2) the thyroid hormone content in the dialysate or ultrafiltrate is measured with a reference measurement procedure. However, in particular because of the former prerequisite, it should be realized that the measurement results will apply exclusively to ED or UF performed under specific experimental conditions. This is because strictly speaking, the equilibrium between free and bound hormone is disturbed to a certain extent during ED or UF, leading to an FTH concentration that is different from the original concentration in undiluted serum. For this reason, the analytically determined values will have to be regarded as approximations of the *real* values fixed by an arbitrarily established set of analytical conditions. On the other hand, it should be recognized that there are measurement procedures such as SD, for which the reverse holds true: disturbance of equilibrium may be minimal to virtually absent. From this perspective, the European Project also puts emphasis on SD with the knowledge that it can never be claimed a reference measurement procedure on its own (see the definition of a reference measurement procedure in Section 4). This is because it is an indirect measurement procedure requiring calibrators with values for the FT4 fraction assigned by ED or UF.

The above described primary aim of the European Project, i.e., the assessment of the validity of ED and UF, fits in the goal of standardizing routine FTH measurements by implementation of a reference measurement system.[26,57] However, in the case that analytical hurdles would prevent unequivocal achievement of such a reference measurement system, it might be of utility that the European Project develops a basis for validation of routine FTH measurements via a measurement procedure that is essentially independent of factors analytically challenging the equilibrium between free and bound hormone, such as is the case for SD. The European Project's lines toward these objectives are described in Appendix C.

7 Clinical Validation of Free Thyroid Hormone Assays

The purpose of clinical validation of FTH assays is to challenge the measurement procedure with a variety of patient specimens representing:

- pathophysiological states;
- biological variation;
- variation of serum binding capacity;
- biological drug effects; and
- physiological preanalytical variables.

7.1 Overview

The primary function of the hypothalamic-pituitary-thyroid axis is to maintain constant—but fluctuating within an individual's narrow limits—levels of FT4, and FT3 in the circulating plasma. The thyroid hormones are transported nearly completely and tightly bound to three binding proteins: TBG, pre-albumin (transthyretin), and albumin. The role of these binding proteins is not limited to transport, since they also assist in maintaining the normal level of circulating FTH, by providing three large thyroid hormone reservoirs.[58]

Table 1 illustrates the relationships of TSH, free and total thyroid hormones, and the binding proteins in the normal euthyroid state, the disease states of hypo- and hyperthyroidism, and a number of pathophysiological situations. The majority of situations have a normal FTH value, which is the goal of the FT4 set point of each individual.[59] This illustrates a defining aspect of FT4 and FT3 assays in that they should be normal or near normal, except in cases of overt thyroid disease and a few unique situations, where their levels should be outside the normal range. Therefore, clinical validation of FTH assays must address the ability of an assay to provide the expected FTH level corresponding to a patient's clinical state.

7.2 Pathophysiological States

7.2.1 The Normal or Euthyroid State

Euthyroidism is usually identified biochemically as normal TSH (~0.4 to ~4.5 mIU/L) and FTH (FT4 ~9 to ~24 pmol/L; FT3 ~3.5 to ~7.0 pmol/L) results, where the FTH data are a reflection of normal levels of total thyroid hormones (TT4 ~58 to ~160 nmol/L; TT3 ~1.2 to ~2.7 nmol/L) and binding proteins.[11] In fact, there are euthyroid states where the total thyroid hormone and binding protein concentrations are outside their individual normal ranges, but in a parallel direction. The most familiar case is pregnancy, where elevations of binding proteins are accompanied by similar increases in the total thyroid hormones to yield normal FTH results (although it should be noted that the TT4 increase is less than the increase in binding proteins, which can result in low/normal FT4 concentrations).[60,61] Conversely, when the binding proteins are depressed in concentration, such as in the low TBG syndromes, the total thyroid hormones are also decreased in concentration, to again yield a normal FTH result.

Table 1. Relationships of TSH, FT4, TT4, FT3, TT3, and Binding Protein Levels

	TSH	FT4	TT4	FT3	TT3	Binding Proteins	
	mIU/L	pmol/L	nmol/L	pmol/L	nmol/L	Conc.	Affinity
Euthyroid	Normal	Normal	Normal	Normal	Normal	Normal	Normal
Disease States							
Mild Hypothyroid	Above Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hypothyroid	High	Low	Low	Low to Normal	Low to Normal	Normal to High	Normal
Mild Hyperthyroid	Slightly Below Normal	Normal	Normal	Normal	Normal	Normal	Normal
Hyperthyroid	Very Low	High	High	High	High	Low to Normal	Normal
Graves' Disease	Very Low	High	High	High	High	Normal	Normal
Special Situations							
Sick Euthyroid	Slightly Low or Normal or High	Low to Normal To High (heparin)	Low to Normal To High (psych illness)	Low to Normal	Low to Normal	Low to Normal	Normal
Secondary Hyperthyroidism (pituitary dependent)	High	High	High	High	High	Normal	Normal
Secondary Hypothyroidism (pituitary dependent)	Normal to Low	Low	Low	Normal to Low	Normal to Low	Normal	Normal
Pituitary Resistance to Thyroid Hormone	Normal	High	High	High	High	Normal	Normal
Pregnancy (High TBG)	Normal to Low	Low to Normal	High	Normal	Normal to High	Elevated TBG	Normal
Low TBG	Normal	Normal	Low	Normal	Low	Low TBG	Normal
TBG Absent Syndrome	Normal	Normal	Low	Normal	Low	No TBG	Normal
Familial Dysalbuminemic Hyperthyroxinemia (FDH)	Normal	Normal	High	Normal	Normal to High	Normal	Albumin affinity is increased for T4.
Thyroid Hormone Displacing Drugs (acute effect)	Normal	High	Normal	High	Normal	Drugs reduce the concentration of binding sites able to bind thyroid hormones.	
T4 Autoantibodies	Normal	Normal	High	Normal	High	Abs in sample that bind T4	
T3 Autoantibodies	Normal	Normal	High	Normal	High	Abs in sample that bind T3	
T4 Replacement Therapy (stable)	Normal (to slightly lowered)	Normal to High (expected to be ~20% higher than upper limit)	Normal (to slightly elevated)	Normal	Normal	Normal	Normal
T4 Suppression Therapy	Low	Normal to High	High	Normal	Normal	Normal	Normal
T3 Thyrotoxicosis	Low	Low to Normal	Low to Normal	High	High	Normal	Normal

7.2.2 Hyperthyroidism and Hypothyroidism

The next group of specimens that must be properly classified is that from patients with overt thyroid disease. In this situation, both TSH and FTH results are outside their respective reference ranges in opposite directions. Hypothyroidism is characterized by elevated TSH results and low FTH results (not FT3, only FT4), while hyperthyroidism is the converse situation, with depressed or undetectable TSH and elevated FTH results. The resulting FTH data are derived from imbalances in the total thyroid hormone levels with respect to the concentrations of the binding proteins.

Mild hypo- or hyperthyroidism, where the TSH is outside the normal range but the FTH is within the population reference range, is a reflection of the log/linear relationship between TSH and FT4. Specifically, there is an amplified pituitary TSH secretion in response to an FT4 abnormality sensed relative to the individual's genetically determined FT4 set point. Currently, the biochemical profile of an abnormal TSH associated with a normal range FT4 value is usually considered to indicate mild thyroid dysfunction. This can occur for both hyper- and hypothyroid disease, as well as from exogenous causes such as L-T4 over treatment, and was previously described as "subclinical."

Patients living in areas of insufficient iodine intake are unable to support the synthesis of T4 in the thyroid. This results in T3 thyrotoxicosis due to the accumulation of T3. In this unique situation, a difference in FTH results is seen. FT4 will be low to normal but FT3 will be elevated, and TSH will also be normal.

7.2.3 Nonthyroidal Illness (NTI)

Also referred to as "sick euthyroid syndrome" or "low T4 syndrome," these specimens come from hospitalized patients who are critically ill, and the physician wants to be sure that there is no underlying thyroid disease. As indicated in Table 1, these specimens may exhibit low, normal, or high TSH and/or TT4 or FT4 values, depending on the stage and severity of illness and the administration of competitive inhibitors of binding proteins or in the case of IV heparin, *in vitro* generation of FFA. In general, TT3 and FT3 values are suppressed. Historically, this specimen population is heterogeneous and has always raised questions regarding the diagnostic accuracy of current FT4 measurement procedures. Due to the variability of FT4 methodologies currently available, the new National Academy of Clinical Biochemistry (NACB) Guidelines[11] recommend in the hospitalized patient population, where low serum binding capacities often occur, the use of TT4 measurements provided that values are interpreted with respect to the severity of illness.[62] The use of an FT4 test in this patient group would require familiarity by the clinical laboratory of the assay performance with their hospitalized patient group.

7.3 Biological Variation

Except for a surge in FTH levels in neonates, circulating levels of the FTHs remain very consistent through the rest of a person's life.[63-67] The exception appears to occur in very late decades of life, where FT4 levels remain constant, but FT3 levels decrease significantly, except in very healthy elderly people.[68,69] There are also only slight, but significant differences in FT4 between men and women, which may be more a reflection of methodological sensitivities to binding protein dependence.[70, 71] During monitoring of maternal thyroid status during normal pregnancy, the elevations in TBG in the mother are paralleled by similar increases in total thyroid hormone, although the FTH levels are usually maintained but vary within the normal range. During the first trimester, maternal FT4 is increased, while TSH is decreased, due to the hCG stimulatory effect on the thyroid. In the third trimester, the FT4 values are lower. However, FT4 and FT3 levels are usually 10 to 15% lower at birth for the mother when compared to nonpregnant women.[60,72] In pregnancies complicated by hyperemesis gravidarum due to extremely high levels of hCG, maternal FT4 is elevated and TSH is suppressed as the woman goes into a transient hyperthyroid state.[73]

7.4 Variation of Serum Binding Capacity

7.4.1 Variations in TBG Concentration

Pregnancy (second and third trimester) is the most frequently observed condition or state in which TBG concentration is elevated from normal levels (due to TBG stimulation by the increased concentration of estrogens). Other situations giving rise to increased TBG concentration (and therefore, increased binding capacity) are estrogen therapies (oral contraceptives or hormone replacement therapy). In contrast, there are situations where the TBG level is lower than normal (partial deficiency)[74,75] or the binding protein is totally absent (complete deficiency).[76] The net effect, in the low or absent TBG situations, is comparably low total thyroid hormone levels, which will yield normal FTH and TSH results. This is a unique example of the normal thyroid state.

Samples containing low or high levels of the binding proteins and, therefore, correspondingly low or high levels of total hormone, should be tested to demonstrate that a normal FTH level is found. This type of study will also demonstrate whether the FTH result is insensitive to changes in levels of binding proteins. In particular, testing low albumin or low total protein specimens will help to verify that the assay performs properly in this clinical state.[77]

7.4.2 Variations in Binding Protein Affinities

There are known familial syndromes where the binding affinity of a binding protein is unusually low or high. Increased binding affinities in albumin have been reported for T4 (familial dysalbuminemic hyperthyroxinemia (FDH), where the albumin affinity towards T4 is increased ~80-fold). There has also been a report of familial dysalbuminemic hypertriiodothyroninemia, where T3 binding by albumin is enhanced, but not T4.[78] The increased binding of the hormone leads to elevations in their total concentrations, but should yield normal FTH results. A measurement procedure for the determination of T4-binding-protein abnormalities is described by Pandian, et al.[79]

There are also situations in which the binding affinity of TBG is reduced, so that the thyroid hormones are less tightly bound.[75] Patients with decreased thyroid hormone binding capacities include hypoalbuminemics, analbuminemics, and patients with decreased or absent TBG and with the low T4 syndrome. This results in decreases in the total thyroid hormone concentrations, but should yield normal FTH levels.

Other documented instances of reduced binding affinities include NTI patients.

NOTE: The FT4 concentrations in pregnancy lie at the bottom of the euthyroid range, whereas patients with the low T4 syndrome lie towards the top of the euthyroid range.

7.5 Biological Drug Effects

Changes in the hormone concentration due to drug-induced physiological responses (i.e., *in vivo* drug interactions) should also be documented and shared with customers. The effects of drugs on FTH measurements are very complex, as reflected by the literature. Compilations of the *in vivo* drug effects on these assays include increases, decreases, and no effects on the laboratory results caused by the same drug.[9,80] There are several reasons for these results. Timing of the serum sampling relative to the initiation of the drug therapy can lead to one set of results, while sampling at the point of maintenance therapy and after conclusion of therapy can lead to different results. Each case is a reflection of the "snapshot in time" of the FTH status of the patient. Further, there are several broad categories of drugs that can interfere with the FTH results, due to physiological effects:

- displacing agents – drugs which compete for binding to the binding proteins: aspirin, clofibrate, danazol, fenclofenac, fenoprofen, furosemide, halofenate, isotretinoin, phenylbutazone;
- iodine rich compounds: amiodarone, iopanoic acid, other radiographic agents;
- inhibitors of thyroid action: methimazole, propranolol, propylthiouracil;
- estrogens – mestranol, norethindrone, other oral contraceptives; drugs which increase synthesis of binding proteins and androgens, and drugs which decrease the synthesis of binding proteins; and
- psychiatric drugs: anticonvulsants, carbamazepine, lithium, phenobarbital, phenytoin.

Specimens drawn from patients taking these medications would be useful for validation, provided the time of the serum sampling and the medication status are known.

7.6 Physiological Preanalytical Variables

The log linear relationship of TSH and FT4 concentrations[81] provides the situation in which the FT4 measurement is the lesser affected of the two markers by physiological preanalytical variables.[82] The FT4 level is not subjected to the influences of circadian or seasonal fluctuations, exercise, and caloric intake, which do affect TSH and FT3.[71,82] TSH can be highly variable, as it responds to minute changes in FT4 in order to re-establish the patient's FT4 set point. This sensitivity makes TSH a more sensitive indicator of thyroid status changes. As mentioned previously, FT4 is generally normal in NTI, while TSH and FT3 are normal or low, respectively.[63] Although the effects of psychiatric illness on thyroid function testing have not been well understood, studies range from reports of abnormal total and FTH levels to one study, which showed that 98% of hospitalized psychiatric patients had normal FT4 levels.[83] Young has collected some of the effects of disease[84] and preanalytical variables[85] on FTH assay results. In the latter case, as with drugs, the effects are reported as having no effect, or a decrease or increase in FTH result, which is a reflection more of how different assays' methodologies are affected. Further, the same preanalytical variable's effect on FTH results has been judged both significant and insignificant for the same magnitude of change by different authors.

7.6.1 Recommendations for Assay Clinical Performance Characterization

The desired performance characteristics of FTH assays, in a variety of situations, have been described above. Clinical validation of the assays requires testing a broad population of specimen types by the manufacturers. Complicating this are the many different types of FTH assays[11] and their differing responses. The more completely the manufacturer demonstrates and reports the performance of the FTH assay, the less would have to be repeated by the laboratorian, which would lead to more appropriate clinical laboratory utilization of the assay.

Two types of clinical validation studies need to be performed. The first, a specimen classification study, determines the FTH assay's response in various clinical conditions. This would include clinically euthyroid, hypothyroid, and hyperthyroid patients with appropriate TSH values. Also to be included are those conditions which alter thyroxine binding protein concentrations, such as pregnancy (with separate validation for each trimester) and renal disease. As these clinical states are relatively common, there should be a sufficient number of subjects in each classification. If available, other conditions which alter the binding proteins should be added to the study. Traditionally, such data have been presented in correlation plots, but scatter plots of the FTH result (y axis) vs. the specimen type (x axis), with the normal range of the assay indicated, may be a better visual presentation. A second FTH assay, either representative of current performance or a reference measurement procedure (e.g., ED or UF), should also be used on the same specimen set for comparison.

The second, a TBG dependence study, documents the FTH assay's insensitivity to TBG and can be done utilizing many of the samples from the previous study. The additional information required would be the total binding capacity of each specimen. A simple plot of the FTH assay result (y axis) versus the total binding capacity (x axis) of the specimens (excluding specimens from hyperthyroid patients) should yield a slope of essentially zero. The binding capacity, range of 0.25 to four times the normal binding capacity (or TBG concentration), should be spanned. This is accomplished by using sick euthyroid specimens (which tend to have low to normal binding capacity due to quantitative or qualitative changes in binding proteins), specimens from pregnant women for high binding capacity, and the normal and hypothyroid patients for normal binding capacity. If specimens of FDH, or TBG-deficient or absent patients, or low and high total protein specimens are available, they should be included. Data for a comparative assay should also be shown.

NOTE: Since any comparison that includes third trimester pregnancy sera will be expected to produce a negative slope (the high TBG samples will have the lowest FT4 values), it might be better to judge the FT4 bias (from the reference measurement procedure) versus the total binding capacity, against the aforementioned acceptance criterion of a slope of zero.

Manufacturers should provide as much performance information to laboratorians as possible. An explanation of what types of specimens have been studied by the manufacturer's measurement procedure, whether performed internally or externally, should be available. Appropriate caveats should indicate if only a limited number of specimens of a particular type were available for testing.

8 Specimen

8.1 Specimen Choice

The specimen of choice is generally considered to be serum.

8.2 Specimen Collection

8.2.1 General

The collection of samples for FT4, in general, should follow the standards set forth in the current editions of NCCLS documents H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* and H4—*Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens.*

8.2.2 Specific for Free Thyroxine

8.2.2.1 Selection of Collection Time

There is no clear evidence that for FT4, time of day or time of year is clinically important.[11]

8.2.2.2 Medications

Ideally, one would withhold all medications that have *in vivo* and *in vitro* effects on FT4 results. Tabulations of these effects are available.[80,85] *In vitro* effects are typically measurement procedure dependent,[11] so it is imperative that the clinician is aware of the response of the measurement procedure the laboratory is using.

8.3 Collection Devices

8.3.1 General

Collection devices should conform to the standards set in the current editions of NCCLS documents H1—*Tubes and Additives for Venous Blood Specimen Collection* and H4—*Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens.*

8.3.2 Additives

In addition to anticoagulants, potential additives to sample collection containers include coating with silicone or thrombin (to promote coagulation when plastic containers are used) and thixotropic gels (to facilitate removal of the sample after centrifugation). As a silicone coating has been reported to interfere with an FT4 assay,[86] healthcare providers should use a device which has been shown, by either the manufacturer or the clinical laboratory, not to create a clinically significant bias in the analytical system being employed. The current edition of NCCLS document EP7—*Interference Testing in Clinical Chemistry* provides a method measuring bias.

8.4 Specimen Processing and Storage

8.4.1 General

Recommendations in the literature for processing and storage of FT4 specimens are conflicting and incomplete.[82,87-90] The laboratory, therefore, should follow the guidelines of the manufacturer of the specific FT4 method used.

8.4.2 Centrifugation

Whole blood collected for serum sampling should be allowed to clot completely before centrifugation. Although serum may remain in contact with a clot for up to 24 hours at room temperature, it is good laboratory practice to centrifuge and separate the serum as soon as possible.

8.4.3 Specimen Storage

General guidelines suggest untested serum/plasma should remain at room temperature for no longer than eight hours, and thereafter, it should be refrigerated at 2 to 8 °C for up to one week.[88] Samples for which the FT4 assay is to be delayed more than one week should be stored at -20 °C. However, if manufacturer guidelines of a specific FT4 assay are available, they should be followed by the user.

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Appendix A. Types of Equilibrium Disturbance

Critical judgment of the design of an FT4 assay should consist of identifying the nature of the disturbance of equilibrium and estimating the magnitude of the resulting differential effects. In order to be able to do so, a mathematical model describing the equilibrium resulting from the interaction between T4 and its binding proteins and inhibitors and/or competitors of T4 binding is required. The model should be simple but adequate, and must not include interaction between binding sites or cooperative effects. The model that is thought to be most useful and robust for describing the FT4 equilibrium is the n ligands, m binding sites model.[1] This model is based on univalent, independent ligands and orders of binding sites on which competition between ligands for binding sites and *vice versa* may occur. It has been successfully applied[2] for the simulation of steroid binding to transport proteins in serum. Descriptions of T4 and T3 binding[3,4] implicitly are based upon this model. With a few additional simplifications, it can be adapted for the description of any FT4 assay system. This includes the action of various types of inhibitors that may be present.[5]

The first simplifying assumption is that all inhibitors present behave like competing ligands of which the free moieties compete with FT4 for unoccupied sites on T4-binding proteins. The net effect is an increase of the dissociation constant for the T4-binding protein equilibrium. Bound inhibitor does not displace T4. Second, an inhibitor may be either dialyzable/filterable or not. "Bound" inhibitors are always considered as being nondialyzable.

In light of this model, a number of typical conditions can be distinguished:

Dilution

1. *The Oppenheimer-Surks model[6] or "ideal" dilution*. From the high affinity of binding proteins for T4 and their concentration, Oppenheimer and Surks predicted that serum FT4 should remain constant when diluting even more than 100-fold. Only at very high dilutions, FT4 will eventually decrease. Mathematically, there is complete analogy with the principle of a buffer solution. In terms of the present model, this is true when there are no inhibitors present of which the free concentration decreases upon dilution, or when the dilution medium contains exactly the same concentrations of all inhibitors as the original serum.

2. *"Nonideal" dilution*. The dilution medium does not contain some or all of the inhibitors of T4 binding normally present in serum. The concentration of unbound inhibitors will decrease proportionally with dilution; free concentrations of loosely bound inhibitors decrease less rapidly because of the buffering effect. Only the competition of inhibitors that are as tightly bound as T4 itself does not change, as their free concentrations change at the same pace as T4. Reduction of free inhibitor concentration accelerates decline of the FT4 concentration. The difference between equilibrium dialysis results for FT4 in chloride-containing and chloride-free buffer, already described by Spaulding[7] in 1972, perfectly illustrates the difference between "ideal" and "nonideal" dilution.

3. *"Perturbing" dilution*. The dilution medium contains substances, extraneous to the serum sample, that inhibit or promote T4 binding to serum proteins or contains T4 binding agents itself. In the first case, a rise of FT4 results; in the other two cases a more rapid decline occurs than with "ideal" dilution.

Ultrafiltration and Equilibrium Dialysis

1. *"Ideal" UF*. In the absence of inhibitors, or in the presence of inhibitors of which the free concentration is constant during filtration (i.e., free inhibitor is filterable), whatever holds for ideal dilution will hold for UF, being the reverse of dilution.

Appendix A. (Continued)

2. *"Ideal" ED*. If no inhibitors are present, or if their (free) inhibitor concentration can be kept constant (dilution/dialysis medium composition), FT4 remains constant like for the ideal dilution case.

3. ED with dialyzable inhibitor present in the serum but not in the dialysis buffer. The FT4 concentration at dialysis equilibrium will be the same as for dilution with the same medium (non-ideal dilution).

4. The presence of nondialyzable/filterable inhibitors constitutes the exception from the more or less generally applicable equivalence of dialysis/UF and dilution. In dialysis/UF, the inhibitor is confined to the dialysand or retentate space, whereas in simple dilution, the total solvent volume is available to the inhibitor. In the latter case, this leads to a lower inhibitor concentration and therefore, to lower FT4.

Binding to Antibodies or Other Proteins

1. If binding protein can be added without affecting the medium (e.g., addition of antibody on a solid support), the conditions of ideal dilution hold. This is because (free) inhibitor concentrations, if present, remain unaltered. There is full analogy between sequestration and ideal dilution. Quantitatively, if the concentration of T4 bound ("sequestered" to the antibody) is equal to the FT4 concentration, this concentration is the same as after "ideal" dilution by a factor two.

2. In routine assays, solid phase supported antibody is often added in suspended form in a medium that contains inhibitors and/or binders of T4. In that case, the combined effects of sequestration (=ideal dilution) and perturbing dilution act on the FT4. In assay validation, it has been common practice to assess sequestration while overlooking perturbing dilution. Some manufacturers even advocate addition of T4 binding protein, especially albumin,[8] either to suppress albumin dependence resulting from the use of labeled analogs or to counteract the effect of FFAs in the sample, or both.

In the only assay for FT4 in which both dilution and unequal distribution of nondialyzable inhibitors can be largely avoided and excluded, namely SD, other aspects of the Law of Mass Action are relevant. First, the assay is based on the assumption of rapid equilibration of free and bound radiolabeled and unlabeled T4 and on equal distribution of these two forms of T4 over free and protein-bound moieties. It is crucial that diffusion through the membrane is the rate-limiting step.

Consequences of the Law of Mass Action

For the sake of clarity, the multiple equilibrium model[1] is reduced to its most basic form, assuming single orders of binding sites for serum proteins and for antibodies used for the assay. A single competitive inhibitor is included and where appropriate, other competing ligands (labeled or unlabelled analogs). The mathematical expressions in this paragraph are consistent with those of Ekins.[9]

The undiluted serum is represented by the following equation:

$$H_0 = F_0 \cdot P_0/(K + F_0 + k_I \cdot I_0) + F_0 \qquad (1)$$

where:

H_0 = Total T4 (concentration)
P_0 = Total binding capacity (concentration)
F_0 = FT4 concentration
I_0 = Inhibitor concentration

Appendix A. (Continued)

K = (dissociation) equilibrium constant
k_I = inhibitor ratio (dimensionless quotient of K and inhibitor dissociation constant).

The sum $K + k_I \cdot I_0$ may be considered as a single "effective" dissociation constant that is dependent on inhibitor affinity and concentration.

The first term in the expression represents the concentration of bound hormone.

Division on both sides by F_0 gives the reciprocal free hormone fraction or "binding index" sometimes advocated[10] as a measure of relative susceptibility of the free hormone to interference in hormone binding.

Ideal Dilution, Differential Effect

The original volume of the sample V_0 is increased to V_t by addition of a medium in which K retains the same value and which contains an inhibitor concentration of I_0. The changes in the total concentrations of hormone and binding protein can be predicted exactly. The free hormone assumes the unknown value F:

$$H_0 \cdot V_0 / V_t = F \cdot P_0 \cdot (V_0 / V_t)/(K + F + k_I \cdot I_0) + F \quad (2)$$

Multiplication by V_t gives the mass balance equation:

$$H_0 \cdot V_0 = F \cdot P_0 \cdot V_0/(K + F + k_I \cdot I_0) + F \cdot V_t \quad (3a)$$

and expressed in terms of concentration:

$$H_0 = F \cdot P_0 /(K + F + k_I \cdot I_0) + F \cdot V_t/V_0 \quad (3b)$$

From (2) follows that for all values of $V_t > V_0$, F must be $< F_0$. Moreover, this difference will be very small if the absolute amount of free hormone ($F \cdot V_t$) is a very small fraction of the total hormone.

Although in Eq. (3) F is not made explicit, it can be understood from this expression that for larger values of V_t, the effect on F will depend on the values of P_0 and I_0 as well. For large values of P_0 and low values of I_0 (i.e., high "binding index"), the effect of increasing V_t on F will be less.

Nonideal Dilution

If the dilution medium does not contain inhibitor, the value of I_0 will decrease by a factor V_0/V_t, which will result in a reduction of the effective dissociation constant. A more rapid decrease of free hormone with dilution will result.

Equilibrium Dialysis

A volume V_0 of the sample is dialyzed against a volume of buffer, so that a total volume of V_t is obtained. At equilibrium, the dialysand (protein compartment) has a volume V_1 (very likely, V_1 will be somewhat larger than V_0 as a consequence of osmotic shift). The dialysis buffer contains an inhibitor concentration of I_0. The inhibitor is dialyzable.

The total amount of hormone concentration remains $H_0 \cdot V_0$. The total protein changes by a factor V_0/V_1. The free hormone assumes the unknown concentration F′ and finally the bound hormone concentration B′ becomes:

Appendix A. (Continued)

$$B' = F' \cdot P_0 \cdot (V_0/ V_1)/(K + F' + k_I \cdot I_0) \quad (4)$$

In order to obtain the mass balance, the free hormone concentration that is present on both sides of the membrane and therefore occupies a volume V_t must also be multiplied by V_t and the bound hormone concentration, which is confined to the dialysand volume V_1 is multiplied by V_1:

$$H_0 \cdot V_0 = F' \cdot V_0 \cdot P_0/(K + F' + k_I \cdot I_0) + F' \cdot V_t \quad (5)$$

Comparison of (5) and (2) shows that F and F′ must be identical.

This identity exists because I_0 is kept constant in both cases. In contrast, if the dialysis buffer does not contain the inhibitor, its concentration changes by a factor V_0/V_t if the inhibitor is dialyzable. The same change will result from dilution with the same buffer without inhibitor. Hence, dialysis and dilution are equivalent as in the case of constant or absent inhibitor. If the inhibitor is not dialyzable, its concentration in ED changes by a factor V_0/V_1. This change may be very small so that the net effect may almost be the same as with ideal dilution, in which the inhibitor concentration is kept constant.

Ultrafiltration

If the serum sample is subjected to UF, a retentate volume V_1 results. The total amount of hormone still is $H_0 \cdot V_0$, the binding protein concentration changes by a factor V_0/V_1, the inhibitor concentration does not change if the inhibitor is dialyzable/filterable, and changes also by V_0/V_1 if it is not. The free hormone concentration acquires the unknown value F″. Finally the bound hormone concentration is:

$$B'' = F'' \cdot P_0 \cdot V_0/V_1 /(K + F'' + k_I \cdot I_0) \quad \text{(inhibitor filterable)} \quad (6a)$$

or

$$B'' = F'' \cdot P_0 \cdot V_0/V_1 /(K + F'' + k_I \cdot I_0 \cdot V_0/V_1) \quad \text{(inhibitor nonfilterable)} \quad (6b)$$

To obtain the mass balance, bound hormone must be multiplied by V_1, free hormone by V_0, the inhibitor concentration by V_1 or V_0 depending on whether it passes through the filter or not:

$$H_0 \cdot V_0 = F'' \cdot V_0 \cdot P_0/(K + F'' + k_I \cdot I_0 + F'' \cdot V_0) \quad (6c)$$

which after division by V_0 is identical to (1), so that $F'' = F_0$. Thus, within the assumptions of the model the true value of free hormone corresponding to the undiluted state can be obtained if no nonfilterable inhibitors are present.

If the inhibitor is nonfilterable, the UF experiment could be arranged in such a way that the final inhibitor concentration in the retentate is equal to the concentration in the undiluted sample. This may be accomplished by prior dilution and continue the filtration process until the retentate volume is equal to the original sample volume. Conditions are now identical to those of ED.

Sequestration and Routine Assays

The starting conditions are those of moderately diluted serum (e.g., 1:5) to a final volume V, characterized by:

$$H = F \cdot P/(K + F + k_I \cdot I) + F \quad (7)$$

Appendix A. (Continued)

Addition of an extraneous binding agent (in routine assays an antibody coupled to a solid support, here denoted by $P_{ab,}$ most often is employed) will result in a new equilibrium and a new value for the free hormone F′. If just antibody is added, the inhibitor concentration does not change:

$$H = F' \cdot P/(K + F' + k_I \cdot I) + F' \cdot P_{ab}/(K_{ab} + F') + F' \quad (8)$$

The second term now represents the concentration of antibody-bound hormone, which clearly is a function (though not linear) of the ambient free hormone concentration.

Transformation to a mass balance and rearranging gives:

$$H = F' \cdot P/(K + F' + k_I \cdot I) + F' \cdot (P_{ab}/(K_{ab} + F') + 1) \quad (9)$$

This expression has the same form as (3b) with $(P_{ab}/(K_{ab} + F') + 1)$ replacing V_t/V_0.

Because the inhibitor concentration is not affected, there is close analogy with ideal dilution. Similar to ideal dilution, if this term is kept small—in the order of a few percent of the total amount—the difference between F and F_a will also be small.

Assessment of Occupancy

1. Two-step, back titration: The total amount of hormone bound to the antibody in the first step will, after removal of the sample, take part in establishment of a new equilibrium in the presence of tracer hormone. The new ambient free hormone assumes the value F″. An expression for the total concentration of tracer hormone H* (labeled T4) is:

   $$H^* = F^* \cdot P_{ab}/(K_{ab} + F^* + F'') + F^* \quad (10a)$$

   The concentration of bound tracer (i.e., the response variable) is represented by the first term of the expression.

   $$B^* = F^* \cdot P_{ab}/(K_{ab} + F^* + F'') \quad (10b)$$

   The term F* in the denominator shows that the tracer in fact competes with ambient T4 for the antibody binding sites. For the sake of assay sensitivity this effect should be minimized, by keeping F* as low as possible. Very effectively, this can be accomplished by replacing labeled T4 by T3 or another analog that has lower affinity (i.e., higher dissociation equilibrium constant K_{ab*}) for the antibody. In that case, Eq. (10b) becomes:

   $$B^* = F^* \cdot P_{ab}/(K_{ab*} + F^* + F'' \cdot K_{ab*}/K_{ab}) \quad (10c)$$

   which shows that the relative influence of the tracer on T4 binding is reduced as K_{ab*}/ K_{ab} increases. This also holds for the following cases.

2. One-step, labeled analog: It is assumed that the presence of labeled analog does not affect (9). The distribution of labeled analog H* is given by:

   $$H^* = F^* \cdot P_{ab}/(K_{ab*} + F^* + F' \cdot K_{ab*}/K_{ab}) + F^* \cdot P/K^* + F^* \quad (11a)$$

Appendix A. (Continued)

The first term is the response variable B*:

$$B^* = F^* \cdot P_{ab}/(K_{ab^*} + F^* + F' \cdot K_{ab^*}/K_{ab}) \quad (11b)$$

The antibody-bound analog tracer clearly depends on the ambient free hormone F′, but may also depend on the binding to serum proteins, represented by the term $F^* \cdot P/K^*$ in (11a). The stronger this binding, the lower the free analog, which occurs at the expense of binding to the antibody (see Appendix B).

3. One-step, labeled antibody: The labeled antibody may bind either to the hormone or to an analog that is coupled to a solid support. The labeled antibody-analog complex now carries the label on the antibody rather than on the analog. Here F_A denotes free analog "concentration" and K_{abA} the dissociation equilibrium constant between analog and labeled antibody. The distribution of the analog shows that also here analog binding to serum proteins would occur at the expense of formation of the labeled complex:

$$H_A = F_A \cdot P^*_{ab}/(K_{abA} + F_A + F' \cdot K_{abA}/K_{ab}) + F_A \cdot P/K_A + F_A \quad (11c)$$

The response variable closely resembles that of the labeled analog approach, with just a different location of the label:

$$B^* = F_A \cdot P^*_{ab}/(K_{abA} + F_A + F' \cdot K_{abA}/K_{ab}) \quad (11d)$$

References for Appendix A

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Appendix B. Theoretical Analysis of the Effect of Analog Binding to Serum Components on Measured Free Hormone

Below is shown that for a particular value of analog tracer binding to the antibody (measurement signal), the corresponding ambient free hormone concentration depends on analog binding in serum (e.g., albumin). The effect is best demonstrated by backward reasoning, starting with two samples yielding the same assay signal but differing in the concentration of T4 and analog (and probably T4) binding protein. Assuming free analog F* is negligible compared to its dissociation constant K_{ab*}, Eqs. (11a) and (11b) can be somewhat simplified and rearranged to:

$$H^* = F^* \cdot (P_{ab} \cdot K_{ab} / K_{ab*})/(F' + K_{ab}) + F^* \cdot P/K^* + F^* \quad (12a)$$

$$B^* = F^* \cdot (P_{ab} \cdot K_{ab} / K_{ab*})/(F' + K_{ab}) \quad (12b)$$

Suppose that two samples, with concentrations of the serum analog binding protein with values P_1 and P_2, produce the same measurement signal B*. Necessarily, the free analog tracer concentration F* will be different in these two cases and takes values of F_1* and F_2*. Since B* is the same in both cases, as is the total analog concentration H*, the relation between F_1* and F_2* is simply:

$$F_1^* \cdot (P_1/K^* + 1) = F_2^* \cdot (P_2/K^* + 1) \quad (13)$$

When substituting in Eq. 12b, it is clear that the ambient free hormone concentration F′ corresponding to signal B* also must differ and have values of F_1' and F_2'. Combining with Eq. 12b and rearrangement gives:

$$(F_1' + K_{ab})(P_1/K^* + 1) = (F_2' + K_{ab})(P_2/K^* + 1) \quad (14)$$

Now suppose that $P_1/K^* = 0.1$, which means that the concentration of analog bound to the serum analog binder is only one tenth of the free analog concentration. If then $P_2/K^* = 0.05$ one obtains:

$$(F_1' + K_{ab})/(F_2' + K_{ab}) = 0.955$$

Measurement ranges usually are of the same order of magnitude as the dissociation equilibrium constant of the ligand with the antibody, so that it is realistic to put $F_1' = K_{ab}$ for an example. Then follows $F_2' = 1.095\ K_{ab}$.

So, with the lower concentration of analog binder, higher free hormone is required to produce the same signal as with the normal concentration, and underestimation of values in samples with lower analog binding results. The same holds for dilution. If sequestration is negligible, ambient free hormone is constant, whereas the concentration of analog binder falls when diluting. The effect is, however, limited to the first dilution steps. In the present example, the maximum underestimation would be 20% and would almost be reached at 1:10 dilution. The analogy can be further extended to the inhibition by FFAs of analog binding to albumin. In this example, the effect could not be more than the said 20%.

A fair solution to the problem of analog binding in serum would be by addition of a binding agent that binds the analog, but not the hormone. This would minimize the effects of variation in the levels of the analog binder without disturbing the equilibrium between free and bound hormone.

Appendix C. The European Project

As described in Section 6, the European Project primarily will explore the feasibility of developing a reference measurement system for FTH measurements, i.e., a certified primary calibrator to realize the SI unit, a reference measurement procedure, and certified matrix reference materials. In parallel, it will validate the procedure of SD. The project concept/lines are described below.

C1 Certified Primary Calibrator

Via another part of the European Project, primary calibrator material for T4 and T3 will be certified for purity. To this end, several commercially synthesized and characterized standard materials will be investigated for purity and presence of specific impurities resulting from synthesis by-products via techniques such as NMR, HPLC, and GC, both combined with different detection principles such as UV and diode array detection, flame ionization detection, and mass spectrometry. It will further be assessed for water content by Karl Fischer titration and for inorganic impurities by ash residue analysis. Based on the outcome of these investigations, a material will be selected with stated impurities and the content for T4/T3 will be certified with its expanded uncertainty.[1] The allowable uncertainty will have to be defined within the project. It should stand in relation to the analytical quality specifications (uncertainty) of the reference measurement procedure(s) (see Section C3). The materials will then be used to prepare via gravimetry calibration solutions for the FTH reference measurement procedure(s).

C2 Reference Measurement Procedure(s)

As mentioned in Section 6, the direct measurement procedures of ED and UF described in Section 5.6 can theoretically be elevated to the status of reference measurement procedures. Nevertheless, it is not yet clear from comparison of results whether ED and UF really are compatible in performance.[2-8] Therefore, it will be the primary objective of the European Project to assess their compatibility. Also of particular importance for ED and UF to become reference measurement procedures is that the thyroid hormone in the dialysate or ultrafiltrate is determined with a reference measurement procedure. The basis for such a procedure will be made available through another objective of the European Project, i.e., the validation of reference measurement procedures for serum TT4 and TT3. According to the project's philosophy, several reference measurement procedures may be used,[9-15] provided they all are based on a common measurement principle fulfilling the highest metrological requirement, i.e., that of a "matrix-independent" measurement principle. ID-MS complies with this requirement.[16] In addition, the reference measurement procedures have to comply with predefined analytical quality specifications and shall be endorsed by authoritative organizations, such as the recently established Joint Committee on Traceability in Laboratory Medicine (JCTLM) (see http://www.bipm.fr/enus/2_Committees/JCTLM.shtml). Therefore, it is the intention of the European Project to assess the compatibility between ED and UF with an ID-MS reference measurement procedure.

In this respect, it should, however, be realized that the requirement of sensitivity (expressed as lower limit of detection) of the reference measurement procedure for thyroid hormone in serum dialysate or ultrafiltrate is of the order of less than 1 pmol/L. This sensitivity might be beyond the reach of current ID-GC or LC/MS, unless large volumes of dialysate or ultrafiltrate are available or unless FT4 is increased artificially by addition of T4. This may seriously limit the reach of an FTH reference measurement procedure. For this reason, the European Project will in parallel put emphasis on SD, because it is supposed that a thoroughly validated SD[17] could play a role in the validation of routine FTH measurements. The SD procedure should be calibrated by ED or UF coupled with ID-MS under such conditions that the sensitivity of the reference measurement procedure is not limiting and should be combined with measurement of the TT4 concentration with a reference measurement procedure.

Appendix C. (Continued)

From this perspective, the compatibility study between ED and UF will start with a simple model consisting of a T4 spiked human serum albumin solution resulting in a relatively high FT4 fraction. This solution will also serve the purpose of calibration of SD. All recommendations with respect to the design and performance of ED (see Section 5.6.1) and UF (see Section 5.6.2) will be strictly adhered to. Other precautions that will be taken are that UF will be done on a diluted sample (dilution with the HEPES buffer, pH 7.4 described in Section 5.5.1), to correspond with the inherent dilution of ED of an undiluted sample. Since it is not yet clear whether a regenerated cellulose membrane with a 3 kDa or 10 kDa cutoff value should be used for UF,[18] both will be assessed. In case of divergence between ED and UF, protein leakage and adsorption to the membrane/surfaces of the UF devices and deterioration of T4 during ED will be investigated as possible causes of the discrepancy. If identical results are obtained by ED and UF, the experiments will be downscaled to the FT4 level as present in hyperthyroid sera. If this downscaling is proven feasible, ED and UF in combination with ID-MS will be used in parallel with SD (calibrated as described before) on real but hyperthyroid sera. Meanwhile, investigations will be continued to improve the sensitivity of the ID-MS reference measurement procedure for serum FTH.

C3 Analytical Quality Specifications of the Reference Measurement Procedures for FTH Measurement

Previously, only the analytical principle of the potential FTH reference measurement procedures has been discussed. However, prior to their development, analytical quality conformance specifications have to be defined. Different models exist to derive them.[19,20] They all basically end up with goals related to the application of the reference measurement procedures. As indicated above, the latter are applied to standardization and/or validation of routine measurement procedures; hence, according to the above philosophy, their analytical quality specifications have to stand in relation to the goals desirable for routine measurement. In the case of FT4, goals, comprising maximum values for bias and imprecision, have been proposed on the basis of biological (intra- and interindividual variation) (see Section 5.2.1) as well as clinical needs.[21] Thus, to guarantee that a reference measurement procedure is adequate for standardization/validation of routine measurement procedures, the European Project will have to come up with superior quality specifications.

C4 Matrix Reference Materials

Matrix reference materials are, as part of a reference measurement system, used for the purpose of standardization and/or validation of routine measurement procedures. Note that they preferably should have their values assigned by a reference measurement procedure. In addition, they should be authentic patient specimens. It is sufficiently known that materials with an artificial or processed matrix (e.g., by delipidation, spiking, lyophilization, etc.) may behave differently from native specimens.[22] Thus, to prevent this phenomenon, referred to as *noncommutability*, both standardization and validation of routine measurement procedures should be done by split-sample measurements with a selected panel of authentic patient samples.

With respect to the implementation of the above approach to routine FTH measurement, it seems that it might be important for the European Project to make a clear distinction between the objectives of standardization and validation. As explained before, it will take time until current ID-MS measurement procedures are optimized to have sufficient sensitivity for application to measurement of thyroid hormone levels in dialysate or ultrafiltrate. Thus, standardization in the strict sense of the word still is not achievable. On the other hand, current ID-MS technology can easily be used to determine the true FT4 fraction in an albumin solution (as described above) intended for calibration of SD. Therefore, until standardization is possible, the assignment of values to matrix reference materials could be done with a properly calibrated and validated SD procedure. In this way, the European Project might tackle the objective of validating routine FT4 measurement procedures. For this purpose, numerical agreement with a reference measurement procedure is less important than the correlation with a measurement procedure that is independent of the

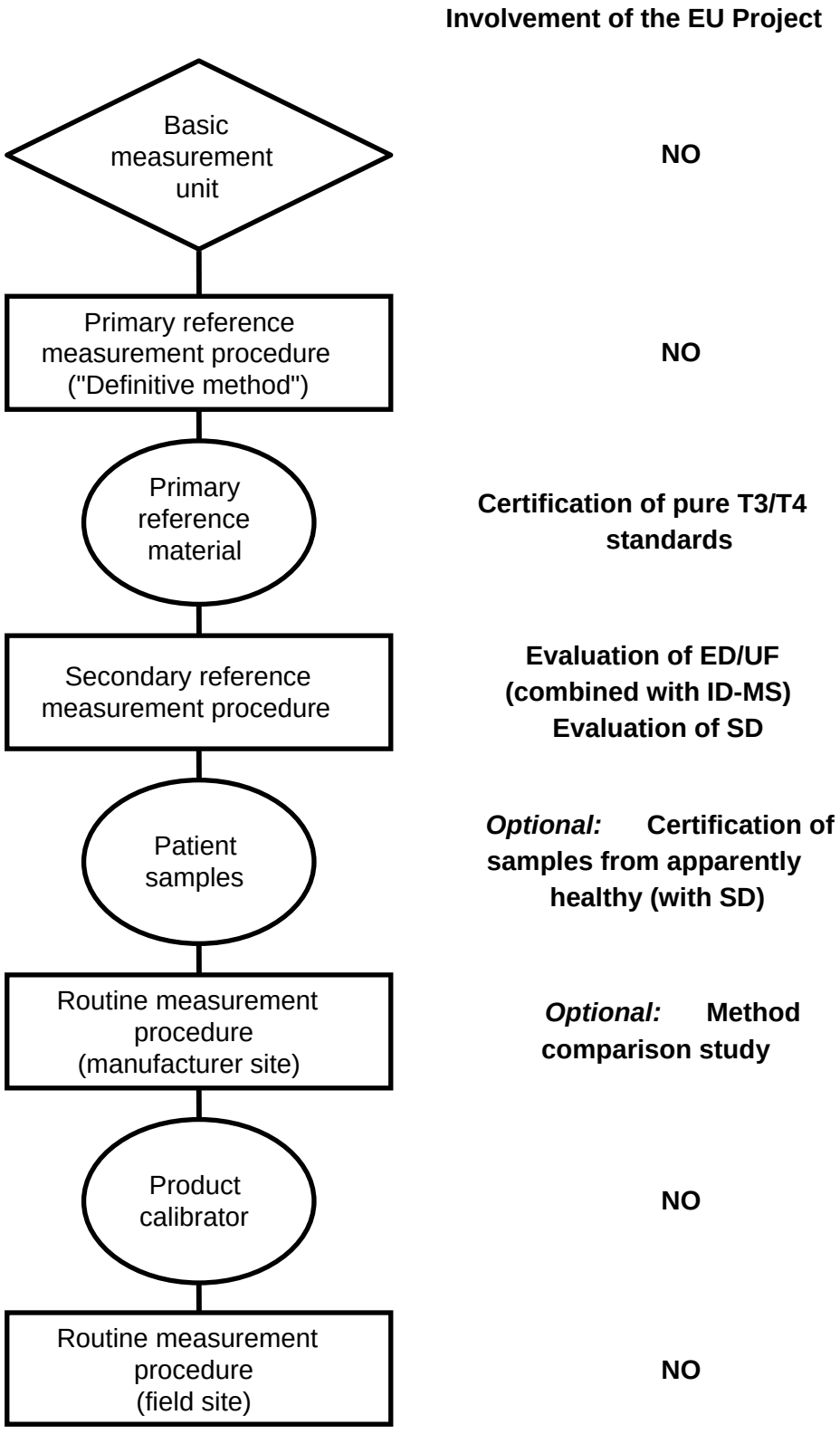
Appendix C. (Continued)

influence of analytically challenging factors, as is the case for SD. It is sufficiently known that in the case of FTH measurement, the dependency of a procedure on serum binding capacity (sBC) (leading to FT4 under- or overestimation) flaws the validity of an assay. Therefore, the patient panel to be used for the aforementioned validation of routine FT4 measurement procedures should include sera whose sBCs and TT4 concentrations cover a wide range and include sera from women using oral contraception or having inherited deficiency or excess of T4 binding proteins, as well as sera from NTI and patients on heparin. The variable impact of these different types of sBC variation is closely related to assay design, particularly if additions have been made to suppress the effect of FFAs.

Appendix C. (Continued)

Overview

The following scheme shows how metrological traceability of a routine measurement procedure is established and where the European Project is involved.



Appendix C. (Continued)

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NCCLS consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact the Executive Offices or visit our website at www.nccls.org.

Summary of Consensus/Delegate Comments and Subcommittee Responses

C45-P: *Measurement of Free Thyroid Hormones; Proposed Guideline*

General

1. A section with guidelines for inclusion and exclusion criteria for establishing reference intervals for FT4 and FT3 would be useful.

- **This is a complex issue which might deserve a separate document.**

  However, for inclusion and exclusion criteria for establishing reference intervals in general, the reader is referred to NCCLS document C28—*How to Define and Determine Reference Intervals in the Clinical Laboratory*.

  For criteria specifically applying to thyroid hormone reference intervals, the reader is referred to studies such as the NHANES III study; see: Hollowell JG, Staehling NW, Flanders WD, et al. Serum TSH, T(4), and thyroid antibodies in the United States population (1988 to 1994): National Health and Nutrition Examination Survey (NHANES III). *J Clin Endocrinol Metab.* 2002;87:489-499. In this study a rather rigorous cut of patients was used by applying specific exclusion criteria such as evidence or clinical suspicion of thyroid abnormalities (e.g., from historical information [including family history] on goiter, thyroid disease, use of thyroid medication), pituitary disorders, drug therapy known to interfere with thyroid gland metabolism, etc.

  A second aspect with respect to FTH reference intervals is that currently a "common" interval is not possible because of the lack of an endorsed reference measurement system to anchor FTH assays. For example, from literature (see, e.g., Demers LM, Spencer CA, eds. *Laboratory Medicine Practice Guidelines: Laboratory Support for the Diagnosis and Monitoring of Thyroid Disease*. National Academy of Clinical Biochemistry [NACB]; 2002), it is obvious that there is closer agreement between the reference intervals of the various ligand assays than there is between the various procedures that employ physical separation. Therefore, awaiting progress in establishing an FTH reference measurement system, we still recommend the establishment of assay-specific FTH reference intervals as very important. This can best be done by a representative sampling of healthy individuals without thyroid disease (on the basis of a "normal" serum thyrotropin concentration, as recommended by the American Thyroid Association; see Ladenson PW, Singer PA, Ain KB, et al. American Thyroid Association guidelines for detection of thyroid dysfunction. *Arch Intern Med*. 2000;160:1573-1575. Erratum in: *Arch Intern Med.* 2001;22;161:284) and by taking the central 95% of the serum FTH concentrations as reference interval for that specific FTH assay.

Section 5.2.1, Imprecision

2. We agree that achieving a 4.8% CV across the entire FT4 assay range is desirable, but this is statistically difficult to attain at the hypothyroid level of < 0.7 ng/mL. The same is true for achieving a 4.0% CV at the hypothyroid level of FT3 (< 2.3 pg/mL).

   FT4 and FT3 are more important in diagnosing hyperthyroidism, and so precision at the low end is not as critical. We do not agree with having one imprecision goal applied across the entire assay range. It should be dependent on the concentration of the analyte and the medical decision level.

- **The committee agrees with the points made and has stated in the text that the within- and between-subject biological variation data in literature are from euthyroid subjects and that the analytical goals apply for the euthyroid level.**

3. The recommendation is that between-lot imprecision and lot-to-lot variability in bias at clinically important doses be available to users. Requiring this extra testing would be a burden for manufacturers.

- **No extra testing should be required as the manufacturer, as part of the QC release testing, assesses each lot manufactured. The information should be made known to any user that requests it.**

Section 5.5.1, Effect of Serum Dilution

4. This section states that "serial dilution (up to an eightfold dilution) of a euthyroid serum with 10 mM hepes pH 7.4 will be expected to reduce the FT4 concentration by no more than 1%." For purposes of interassay comparison, will the dilution factor of the sample in the total reaction volume be the accepted starting point for the serum dilution profile, as was the case for your example in Figure 2? If so, for assays which have a large sample dilution factor (i.e., 1 to 25 in the case of our FT4 assay) an eightfold dilution factor would equate to a final dilution factor of 200.

   If an assay were to successfully meet the two other validity criteria outlined in Sections 5.5.2 and 5.5.3, then we would like to recommend that a deviation of <10% for a twofold dilution (which would equate to a 50-fold dilution factor) is a reasonable performance check for the serum dilution test.

- **Serum dilution is used to predict the performance of the assay in patient sera that have different sBC. The diluting of a serum with an inert buffer makes different sBC sera (these should be viewed as patient-mimics). The dilution factor in the assay is irrelevant. What is important is finding out whether a sample with low sBC is biased by a particular kit or not. An assay that utilizes a high serum dilution is expected to be negatively biased in low sBC sera.**

Section 5.5.3, Addition to Serum of Substances that Cause Displacement of Thyroid Hormones From Their Binding Proteins

5. It is stated that "the effect of these drug interactions should be compared to those obtained in the reference measurement procedure (when this is available)."

   a) There is no established reference method for free hormones, as stated in Section 5.6: "Unfortunately, there is currently no officially endorsed reference measurement procedure."

   b) Equilibrium dialysis is not commercially available for FT3 measurement.

- **The point made is correct; however, since reference measurement procedures are being developed and will be available soon, the text is retained with the constraint regarding availability of a reference measurement procedure.**

Section 7.6.1, Recommendations for Assay Clinical Performance Characterization

6. The clinical validation studies proposed (clinically euthyroid, hypothyroid, hyperthyroid, pregnancy samples at each trimester, and renal disease patients with 30 subjects in each classification) is beyond what a manufacturer should do. These studies would require informed consent and chart review and would be very costly.

- **The proposed clinical validation is supported by Guideline 16 (For Manufacturers: Assessment of FT4 Estimate Test Diagnostic Accuracy) of the National Academy of Clinical Biochemistry (NACB) document (see Demers LM, Spencer CA, eds. *Laboratory Medicine Practice Guidelines: Laboratory Support for the Diagnosis and Monitoring of Thyroid Disease*. National Academy of Clinical Biochemistry [NACB]; 2002). We agree that this validation is a difficult and expensive task for the manufacturer; however, looking at samples, which challenge an immunoassay's performance, is the sole way to determine its validity. A manufacturer who does not have this type of validation work in the documentation for the assay will be challenged by the routine customers as well as by the FTH experts on the clinical validity of the test.**

Nevertheless, the text has been modified to not specify the number of patients in each category.

Section 8.4.1 General

7. "Recommendations in the literature for processing and storage of FT4 specimens are conflicting and incomplete. The laboratory, therefore, should follow the guidelines of the manufacturer of the specific FT4 method used." This statement is contradictory to the statement in Section 8.4.3, which outlines the specimen storage guidelines for FT4.

- **The committee agrees with the point made and has modified the text in Section 8.4.3.**

NOTES

The Quality System Approach

NCCLS subscribes to a quality system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents through a gap analysis. The approach is based on the model presented in the most current edition of NCCLS HS1—*A Quality System Model for Health Care.* The quality system approach applies a core set of "quality system essentials (QSEs)," basic to any organization, to all operations in any healthcare service's path of workflow. The QSEs provide the framework for delivery of any type of product or service, serving as a manager's guide. The quality system essentials (QSEs) are:

Documents & Records	Equipment	Information Management	Process Improvement
Organization	Purchasing & Inventory	Occurrence Management	Service & Satisfaction
Personnel	Process Control	Assessment	Facilities & Safety

C45-A addresses the quality system essentials (QSEs) indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Documents & Records	Organization	Personnel	Equipment	Purchasing & Inventory	Process Control	Information Management	Occurrence Management	Assessment	Process Improvement	Service & Satisfaction	Facilities & Safety
					X EP7						M29

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, GP26-A2 defines a clinical laboratory path of workflow which consists of three sequential processes: preanalytic, analytic, and postanalytic. All clinical laboratories follow these processes to deliver the laboratory's services, namely quality laboratory information.

C45-A addresses the clinical laboratory path of workflow steps indicated by an "X." For a description of the other NCCLS documents listed in the grid, please refer to the Related NCCLS Publications section on the following page.

Preanalytic					**Analytic**		**Postanalytic**	
Patient Assessment	Test Request	Specimen Collection	Specimen Transport	Specimen Receipt	Testing Review	Laboratory Interpretation	Results Report	Post-test Specimen Management
	H3	H1 H3 H4	H18	H18				

Adapted from NCCLS document HS1—*A Quality System Model for Health Care*.

Related NCCLS Publications*

EP6-A **Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline (2003).** This document provides guidance for characterizing the linearity of a method during a method evaluation; for checking linearity as part of routine quality assurance; and for determining and stating a manufacturer's claim for linear range.

EP7-A **Interference Testing in Clinical Chemistry; Proposed Guideline (2002).** Provides background information, guidance, and experimental procedures for investigating, identifying, and characterizing the effects of interfering substances on clinical chemistry test results.

EP9-A2 **Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Second Edition (2002).** This document addresses procedures for determining the bias between two clinical methods or devices and for the design of a method comparison experiment using split patient samples and data analysis.

H1-A5 **Tubes and Additives for Venous Blood Specimen Collection; Approved Standard—Fifth Edition (2003).** This standard contains requirements for blood collection tubes and additives including heparin, EDTA, and sodium citrate.

H3-A5 **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. It also includes recommendations on order of draw.

H4-A5 **Procedures and Devices for the Collection of Diagnostic Capillary Blood Specimens; Approved Standard—Fifth Edition; (2004).** This document provides a technique for the collection of diagnostic capillary blood specimens, including recommendations for collection sites and specimen handling and identification. Specifications for disposable devices used to collect, process and transfer diagnostic capillary blood specimens are also included.

H18-A2 **Procedures for the Handling and Processing of Blood Specimens; Approved Guideline—Second Edition (1999).** This guideline addresses multiple factors associated with handling and processing specimens, as well as factors that can introduce imprecision or systematic bias into results.

M29-A2 **Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline—Second Edition (2001).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.

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The Nebraska Medical Center
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New England Medical Center (MA)
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Sunrise Hospital and Medical Center (NV)
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