REVIEW

Clinical Utility and Analytical Aspects of Direct Measurements of Free Hormones Using Mass Spectrometry-Based Methods

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Background: The free hormone (FH) hypothesis states that hormone action and the corresponding biological effects are mediated by the unbound (free) fraction of hormone in circulation. The in vivo relationship between protein-bound and FH is complex and dynamic. In most individuals, measurement of total hormone (TH) is usually adequate to reflect the hormone status; however, certain physiological conditions and/or medications can affect protein binding and alter FH concentration. In these cases, measurement of FH will provide a better measure of the bioactive hormone status than measurement of the TH. Measurement of FH presents many challenges, as the concentrations are very low and there are number of pitfalls, which may affect the measured concentrations.

Content: In this review, we discuss techniques used in the separation and direct quantitation of FH concentrations in biological samples using mass spectrometry for analysis. We also highlight clinical situations in which FH analysis is warranted and when mass spectrometry should be the preferred methodology over immunoassays.

Summary: Equilibrium dialysis, ultrafiltration, or size-exclusion separation coupled with liquid chromatography–tandem mass spectrometry provides a sensitive and specific method to measure FH concentrations. These direct methods are useful in iatrogenic or physiological states that alter hormone binding or metabolism.

INTRODUCTION

Circulating plasma hormones are predominantly bound to carrier proteins, enabling hormone solubilization, stability, reserve, and transport to facilitate and maintain endocrine signaling. Hormones exist in equilibrium with tight-binding proteins (carrier proteins), loosely bound proteins (e.g., albumin), and a small fraction that is nonprotein bound (free). Physiologic response to hormones occurs when the hormone enters target tissues and mediates its actions at the cellular level (1, 2). Entry into most cell types is limited to the free- and loosely bound hormones, i.e., the "free hormone hypothesis" (3, 4). Regulation of free hormone (FH) activity and downstream effects is a function of binding protein concentration, binding affinity, and total hormone (TH) concentrations; governed by feedback mechanisms involving appropriate endocrine axes. While the FH hypothesis is accepted by most clinical

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IMPACT STATEMENT

Measurement of free hormone (FH) concentrations in biological samples presents a challenge to the clinical laboratory. FH concentrations are generally very low, requiring use of sensitive and specific techniques. Furthermore, special attention must be placed on the equilibrium between free and protein-bound hormone when separating and analyzing in vitro. This review will enhance the readers' understanding of the current state of mass spectrometry-based methods for the measurement of FHs. The advantages and disadvantages of different separation techniques and sample preparation methods are discussed, as well as clinical conditions in which measurement of FH is warranted.

practitioners, basic research and clinical studies continue to better define when measurement of TH or FH is most clinically relevant to a particular disease state for diagnosis, treatment, and monitoring (1, 5).

Some physiologic conditions, medications, and acquired or inherited carrier protein abnormalities can cause atypical fluctuations of TH and FH concentrations. In these scenarios, when a patient has conditions influencing equilibrium between hormones and their binding proteins, concentrations of free or bioavailable (sum of free and loosely bound hormone) hormone could be more informative than TH concentrations (6–8). Because FHs are present in biological samples in trace concentrations, highly sensitive and specific methods are required for accurate measurement of FH concentrations.

Since the 1970s, several FH analytical methods have been developed, spanning indirect estimation by calculation (derived from measurements of TH, binding protein concentrations, and associated binding constants), to direct quantitation of isolated FH. For a detailed review of approaches to measure FHs, the reader is directed to previous reviews on the subject (3, 9). A limitation of calculation-based methods is in the assumption of uniform hormone affinity to binding proteins among individuals. However, binding protein variants, posttranslational modifications, and other factors may alter binding affinity, causing erroneous results and biases (10). Direct immunoassay methods show discrepancy among methods of different manufacturers, and often perform poorly when binding protein concentrations are very elevated or decreased, with some methods suffering from biotin interference (9). Continual method improvement, harmonization, and standardization efforts have been described in literature over the last 40 years, resulting in the formation of guidance documents (11) and a reference measurement procedure for free thyroxine (FT4) (12). Analyses based on FH separation from biological samples by equilibrium dialysis are often referred to in the literature as a reference method, while that term should not be confused with reference measurement procedures (i.e., definitive methods) that are used to validate metrological traceability of analytes in reference methods. Reference methods for free plasma cortisol, free testosterone (FTe), free estradiol (FE2), FT4, free triiodothyronine (FT3), and free 25-hydroxy vitamin D (F25OHD) are described throughout this review.

To standardize testing and to harmonize among-laboratory quantitative agreement, it is important to use high quality reference materials for preparation of the calibration standards used in methods for measurement of FHs. Certified reference materials for FHs of clinical interest are

available from some commercial companies and government institutions, including Ceriliant, the National Institute of Standards & Technology, European Commission Institute for Reference Materials, Measurements, and the National Measurement Institute.

Limitations of immunoassay-based methods have prompted consensus group recommendations to extend the use of reference methods to test patient specimens for particular clinical scenarios, e.g., the management of thyroid disease during pregnancy (13), in individuals with protein-losing disorders (14), or when a binding protein abnormality is suspected (15). Equilibrium dialysis (ED) followed by LC–MS/MS is considered the gold standard methodology, with a number of LC–MS/MS methods developed and introduced in routine patient testing (12, 16–22).

While recently published reviews on the state of clinical FH measurements state that mass spectrometry-based methods are incompatible with high-throughput testing (3, 23), high-throughput methods for measurement of all diagnostically important FHs have been developed and introduced in routine use at many clinical reference laboratories (15, 21, 22, 24–26).

Our aim of this review was to detail established and novel techniques for direct measurement of small molecule FHs with subsequent analysis by mass spectrometry, and to provide reference tables describing known variables affecting FH concentrations in vivo and in vitro. Last, we sought to provide references for clinical scenarios when FH analysis by direct methods using mass spectrometry detection is recommended for accurate measurement of FHs in clinical practice.

CLINICAL CONSIDERATIONS FOR FREE HORMONE EVALUATION

General TH measurements are a frequent component in the primary evaluation of many common disorders, or in the monitoring of medical treatment. However, in clinical states of altered binding protein concentrations or presence of the binding protein variants, TH concentrations may incorrectly suggest overt endocrine disfunction. For instance, a falsely elevated TH result may lead to an overestimation of hormone action and subsequent underestimation or underdiagnosis of poor endocrine function (14, 23, 27, 28).

Free Hormones and the Physiological State

Overt reduction in gland function or stimulation leads to reduced TH and FH plasma concentrations, and conversely, excess gland stimulation or ectopic hormone production leads to increased concentrations of TH and FH (Table 1). In health, the hypothalamic-pituitary-end organ axes promote homeostasis by adjusting TH and binding protein concentrations to optimize FH concentrations needed for physiological demands. Therefore, TH and FH ratios are impacted when physiological states influence binding protein concentration or affinity for the target hormone (10). Binding protein excess or deficiency, structural variants, or altered binding affinity in some cases could be compensated, to allow for normal concentrations of FHs (15). Table 1 also depicts how conditions other than those affecting primary or secondary endocrine glands, such as pregnancy, renal insufficiency, obesity, liver disease, aging, and malnourishment, influence binding protein concentration, function, and the hormone metabolism, often result in altered FH concentrations and pathologic changes.

Medication Influence on Free Hormone Concentrations

Medications may directly influence the production or suppression of hormones by their direct (i.e., on-target) influence on a specific endocrine signaling axis (Table 2). TH and FH concentrations adjust appropriately to these medications when the endocrine axis is intact as the body alters the production of binding proteins to adequately

Table 1. Clinical conditions and binding protein abnormalities affecting in vivo free hormone concentrations.

Condition	Free hormone	Expected change	Mechanism	References
Overt hypothyroidism	FT3, FT4	Decrease	Reduced production of thyroid hormones	(29)
End stage renal disease	FT3	Decrease	Reduced conversion of T4 to T3	(30)
Overt hyperthyroidism	FT3, FT4	Increase	Increased production by the thyroid or excess thyroxine treatment	(31)
Pregnancy	FPC	2–3-fold increase	Increased CBG concentration and placentally-derived CRH. Also, there is a shift of CBG binding to cortisol to progesterone as concentrations rise during pregnancy.	(32)
	FT3, FT4	(a) Slight decrease in second and third trimesters (b) Increased in hyperemesis gravidarum	(a) Estrogen-induced increase in TBG (b) Due to very high concentrations of hCG, FT4 is elevated	(33)
	FTe	TTe increases initially followed by an increase in FTe late in pregnancy	Initially decreased metabolic clearance followed by increased production. Increased SHBG concentration normalizes FTe early in pregnancy	(34)
Liver disease	FTe	Decrease	Lowered TTe and increased SHBG	(35)
	FT3	Decrease	Decreased FT3 in acute and chronic liver disease. Likely due to changes in binding proteins, and hormone metabolism.	(36)
	FPC	Baseline decrease and low concentrations post stimulation	Impaired synthesis, cholesterol abnormality, and increased cytokines	(37)
	F25OHD	Increased	Decreased VDBP	(38)
Adrenal insufficiency	FPC	Decrease	Decreased in vivo synthesis of cortisol	(39)
Preeclampsia and gestational hypertension	FPC	Below normal in third trimester	Lower than normal CBG and HPA axis response	(40)
Critical illness	FPC	Increase	Significant change in CBG, decrease metabolism of cortisol, altered protein binding, and peripheral cortisol synthesis	(7, 41)
	FTe	Decrease	Mechanism not well defined	(42)
PCOS	FE2 (limited evidence)	Increase	Hyperinsulinemia reduces SHBG and elevates LH	(43)
	FTe	Increase		(44)
Obesity/BMI	FE2 (limited evidence)	Increase in males and post-menopausal females	Obesity reduces SHBG and there is increased peripheral conversion of Te to estrogens	(45)
	FT4	Decrease	FT4 correlates inversely with BMI	(46)

Continued

Table 1. (continued)

Condition	Free hormone	Expected change	Mechanism	References
	FTe	Increase in females, decrease in males	Obesity reduces SHBG and peripheral conversion of Te to estrogens	(45)
	FPC	Increase	Severe obesity can cause activation of the HPA axis	(47)
Hirsutism	FTe	Increase	TTe is often normal, but due to decreased plasma SHBG, FTe increases	(48)
Aging	FTe	Decrease	FTe and TTe production decrease with age in men. FTe may decrease at a faster rate due to increasing SHBG with age. FTe is diminished in women in years prior to and in menopause	(49)
	F25OHD	Increase	Increased total vitamin D and decreased VDBP in individuals over 65 y.o.	(50)
	FT4	Increase	Decrease in TSH, primarily in individuals over 65 y.o.	(46)
Hypogonadism	FTe	Decrease	Decreased production of Te, increase in SHBG expression and increased E2	(51)
Premenstrual dysphoric disorder	FE2	Decrease	Increased plasma SHBG	(52)
Thyroid hormone resistance	FT3	FT3: increase	Inactivating mutations of TSH or TRH receptors	(53)
	FT4	FT4: increase		
Autoimmunity	FT4	Increase or decrease	Stimulating or blocking autoantibodies induce changes in TSH and FT4	(46)

Body mass index (BMI), corticotropin-releasing hormone (CRH), cortisol binding globulin (CBG), estradiol (E2), free estradiol (FE2), free plasma cortisol (FPC), free testosterone (FTe), free thyroxine (FT4), free triiodothyronine (FT3), human chorionic gonadotropin (hCG), hypothalamus-pituitary-adrenal (HPA), luteinizing hormone (LH), poly cystic ovary syndrome (PCOS), sex-hormone-binding globulin (SHBG), thyroid peroxidase (TPO), thyroid-stimulating hormone (TSH), thyrotropin releasing hormone (TRH), thyroxine-binding globulin (TBG), total testosterone (TTe), vitamin D binding protein (VDBP).

compensate (6). Medications can also have an indirect (i.e., off-target) influence on TH and FH concentrations, often by their influence on the principal binding protein, either by altering protein expression or binding affinity (70). Transient fluctuations of FH can occur in vivo based on drug dosage, drug-specific pharmacokinetics, and timing of administration. It is prudent to interpret test results considering the medications administered to the patient and time of specimen collection (71). When FH concentrations in these individuals do not correlate with clinical findings, assay-specific limitations, and preanalytical factors should be considered.

As detailed in Table 3, isolation of the FH and subsequent measurement by mass spectrometry are often warranted to improve analytical accuracy and specificity of FH analyses in patient specimens where a physiologic state or medication use may interfere with conventional methods. Established and promising new techniques for direct measurement of various FHs are detailed next and summarized in Fig. 1 and Table 4.

Table 2. Medication influence on physiology and binding proteins, affecting in vivo or in vitro free hormone concentrations.

Hormone	Medication	Observed change	Mechanism	Physiologic or analytical change[a]	References
FPC	Cortisol stimulating agents (e.g., cosyntropin)	Increase in individuals with intact HPA axis	Therapy affects the HPA axis to increase FPC	Physiologic	(54)
	Cortisol suppressing agents (e.g., metyrapone, dexamethasone)	Decrease in individuals with intact HPA axis	Therapy affects the HPA axis to decrease FPC	Physiologic	(55)
	Exogenous glucocorticoid therapy	Increase or decrease	Hydrocortisone (i.e., medicinal cortisol) will increase FPC while other synthetic glucocorticoids can suppress cortisol production	Physiologic	(56)
	Oral contraceptives	Increase	Estrogen therapy increases CBG, total cortisol, and FPC	Some immunoassays result falsely low cortisol with elevated CBG. Elevations of free and total cortisol observed in vivo	(14, 54)
FE2	Oral contraceptives	Increase	Exogenous hormone contribution	Physiologic	(32, 57)
	Opioids (oxycodone, hydrocodone, morphine), long-term use	Decrease	Decreased GnRH secretion and therefore reduced LH	Physiologic	(58)
	Anticonvulsants (e.g., phenytoin, carbamazepine, phenobarbital, valproic acid)	Decrease	Increased plasma SHBG	Physiologic	(59)
FT3 and FT4	Oral contraceptives	FT3 Increase	Low plasma TBG	Some immunoassays result falsely elevated FT3 when TBG is low	(14)
	Heparin, salicylates, phenytoin, carbamazepine, furosemide, phenobarbital, phenytoin, NSAIDs	Increase	Displacement of T3 and T4 from binding proteins	Transient in vivo with normalized FH concentrations over time. In vitro, drugs may affect FH measurements in some assays.	(60, 61)
	Amiodarone	Increase (in AIT) Decrease (in AIH)	(a) Increased iodine exposure to the thyroid (b) Inhibition of 5′-monodeiodination of T4	Physiologic	(62)
	Lithium, tyrosine kinase inhibitors, antithyroid agents	Decrease	Thyroid toxicity, inflammation	Physiologic	(63)

Continued

Table 2. (continued)

Hormone	Medication	Observed change	Mechanism	Physiologic or analytical change[a]	References
	Anticonvulsants (e.g., phenytoin, carbamazepine, phenobarbital, valproic acid)	FT4 decrease	Altered T4 metabolism	Physiologic	(63)
	Inorganic Iodide/Iodine	Increase or decrease	Transient changes to thyroid function. Impairment (e.g., Wolff–Chaikoff) or induced hyperfunction (e.g., Jod–Basedow)	Physiologic	(64, 65)
FTe	Oral contraceptives	Decrease	Increased plasma SHBG	Physiologic	(32, 57)
	Corticosteroids	Decrease	Suppression of GnRH secretion	Physiologic	(32, 66)
	Synthetic PPAR-gamma ligands (thiazolidinediones)	Decrease	Increased plasma SHBG	Physiologic	(32, 67)
	Ethinyl estradiol	Decrease	Increased plasma SHBG and suppression of LH and FSH	Physiologic	(58)
	Anticonvulsants (e.g., phenytoin, carbamazepine, phenobarbital, valproic acid)	Decrease	Increased plasma SHBG	Physiologic	(59)
	Metformin	Decrease	Increased plasma SHBG	Physiologic	(68)
	Danazol	Increase	Decreased plasma SHBG	Physiologic	(69)

Amiodarone-induced thyrotoxicosis (AIT), Amiodarone-induced hypothyroidism (AIH), cortisol binding globulin (CBG), follicle stimulating hormone (FSH), free estradiol (FE2), free hormone (FH), free plasma cortisol (FPC), free testosterone (FTe), free thyroxine (FT4), free triiodothyronine (FT3), gonadotropin releasing hormone (GnRH), hypothalamus-pituitary-adrenal (HPA), luteinizing hormone (LH), nonsteroidal antiinflammatory (NSAID), sex-hormone-binding globulin (SHBG), thyroxine (T4), thyroxine-binding globulin (TBG).

[a] Binding protein concentration changes generally correlate with TH concentrations while FH generally remain within reference intervals. Some immunoassays are susceptible to inaccurate measurements of FH when binding proteins are substantially altered.

Table 3. Conditions and potential interferences, warranting free hormone analysis by mass spectrometry.

Clinical condition/scenario	Analyte	Clinical need	Rationale for mass spectrometry testing	References
Hypothyroidism	FT3 FT4	To appropriately distinguish between hypothyroidism and subclinical hypothyroidism	1. FT4 IA nonlinearity at low analyte concentrations. 2. Some patients may exhibit signs and symptoms of hypothyroidism while treated with levothyroxine even though TSH is normal. 3. Genetic variants of TBG 4. Medications can influence FT3 and FT4 IA measurements.	(6, 28, 71)
Testosterone status in females and children	FTe	In women with hyperandrogenemia, free testosterone correlates better with clinical presentation than total testosterone	Precision and accuracy issues at low testosterone concentrations. Testosterone measured by LC–MS used in conjunction with SHBG concentrations to determine free testosterone by calculation.	(5)
Hypogonadism	FTe and FE2	Recommended to distinguish eugonadism from hypogonadism. Also, useful to monitor hormone suppressing therapies and to evaluate hormone status in individuals with protein-binding abnormalities	Equilibrium dialysis is reference method, may be useful when calculated values of FH do not correlate with clinical presentation.	(51)
Hyper and hypoadrenalism	FPC and cortisol	Protein binding of serum cortisol is highly variable due to nonlinear molecular binding and inter- and intra-individual variation in CBG concentration	When CBG levels are >95th percentile, the differences between total cortisol IA and mass spectrometry results are statistically significant. Elevated CBG is strongly associated with use of oral contraceptives. IAs underestimate total cortisol when CBG is elevated.	(14, 54, 72)

Continued

Table 3. (continued)

Clinical condition/scenario	Analyte	Clinical need	Rationale for mass spectrometry testing	References
			During acute illness, CBG concentrations decrease substantially, so FPC will increase despite constant total concentration. When clinical symptoms are inconsistent with total cortisol results, FPC by mass spectrometry is warranted.	
Thyroid function during pregnancy—central hypothyroidism or hormone treatment monitoring—fetal risk of FT4 deficiency	FT4	Hypothyroidism occurs in approximately 2.5% of pregnant women (73). Fetal dependency <12 weeks gestation (74)	FT4 IAs are inaccurate at extremes of binding protein concentration. ED methods show lower third trimester FT4 levels than nonpregnant females. Note that there is lacking consensus of FT4 method use during pregnancy.	(13, 28, 73–77)
Nonthyroidal illness	FT3, FT4	Accurate thyroid assessment during critical illness is necessary for treatment	Significant changes in binding protein capacity during acute illness. FT4 and FT3 IA interference may not allow for distinguishing between nonthyroidal illness and true hypothyroidism. Drugs and other shifts in hormone to protein binding alters FT4 and FT3 concentrations. NACB guidelines suggest total T3/T4 measurements may be more suitable.	(78, 79)
Thyroid hormone-binding protein abnormalities	FT4	Individuals with FDH are often euthyroid but found with false elevations of FT4	Falsely elevated concentrations are observed in some FT4 IAs, caused by abnormalities in the binding proteins (e.g., congenital TBG deficiency, FDH).	(6)

Continued

Table 3. (continued)

Clinical condition/scenario	Analyte	Clinical need	Rationale for mass spectrometry testing	References
Renal failure, nephrotic syndrome and hemodialysis	FT4	Thyroid function is altered in chronic kidney disease, often requiring treatment and monitoring	There is evidence of binding protein inhibitors present in patients with ESRD these factors may inhibit in vivo binding of T4 to TBG in vivo, and in vitro assessment in some FT4 IAs.	(80)
Presence of endogenous autoantibodies	FT3, FT4	To evaluate nonsuppressed TSH when THs are elevated by FT4 or FT3 IAs	Erroneous thyroid FH results may be due to autoantibody interference. To be considered when other forms of assay interference, identification of binding protein abnormalities, and evaluation of resistance to thyroid hormone and TSH-secreting pituitary adenoma have been ruled out.	(81, 82)
Biotin therapy	FT3, FT4	To evaluate thyroid function in patients receiving high-dose biotin therapy	Competitive FT4 and FT3 IAs that use biotin to streptavidin binding in the presence of patient sample are susceptible to interference.	(83)
Known or suspected heterophile or human antianimal antibodies	FT3, FT4	General interference to be considered when IA results are not consistent with clinical evidence	Heterophile antibodies can react with animal immunoglobulins used in FT3 and FT4 IAs.	(82)
Critical illness	FPC FTe	Critically ill patients often have adrenal insufficiency and hypotestosteronemia	Critical illness may impact binding protein concentrations and free hormone concentrations and direct measurement of FH in plasma may better correlate with clinical evidence.	(7, 41, 42, 84)
Adrenal insufficiency assessment in patients with binding protein derangements	FPC	Accurate assessment of adrenal insufficiency is aided by measuring free plasma or serum cortisol	Discrepancies between total cortisol and FPC measurements can confound adrenal insufficiency diagnosis in patients with abnormal binding protein profiles as a result of liver disease, medication use, and critical illness.	(72, 85)

Continued

Table 3. (continued)

Clinical condition/scenario	Analyte	Clinical need	Rationale for mass spectrometry testing	References
Free vitamin D assessment in patients with liver disease or kidney disease, elderly, pregnant women, and ethnicities with different genotypes of VDBP	F25OHD	VDBP abnormality to be considered when F25OHD results obtain by estimation methods are not consistent with clinical evidence	Discrepant VDBP values are observed when measured by total 25-OH-vitamin D IAs. These discrepancies confound estimations of F25OHD in individuals with VDBP abnormalities or genotype variants.	(86–89)

Cortisol binding globulin (CBG), equilibrium dialysis (ED), familial dysalbuminemic hyperthyroxinemia (FDH), follicle stimulating hormone (FSH), free estradiol (FE2), free hormone (FH), free plasma cortisol (FPC), free testosterone (FTe), free thyroxine (FT4), free triiodothyronine (FT3), immunoassay (IA), National Academy of Clinical Biochemistry (NACB), testosterone (Te), thyroid-stimulating hormone (TSH), thyroxine (T4), thyroxine-binding globulin (TBG), total hormone (TH), vitamin D binding protein (VDBP).

SEPARATION OF FREE ANALYTES FROM BIOLOGICAL SAMPLES

Three techniques have been used to date for separation of nonprotein-bound hormones and drugs from biological samples, i.e., ED, ultrafiltration (UF), and size-exclusion separations (SES) (3, 8, 9, 92, 93).

Equilibrium Dialysis

Dialysis is a process of separating molecules based on their size by diffusion through a semipermeable membrane, placed between a chamber containing biological sample and a chamber with a dialysis buffer. During the dialysis process, molecules diffuse through the membrane from an area of higher concentration to the area with lower concentration, until an equilibrium is established.

Dialysis membranes are typically made of cellulose, modified cellulose, or synthetic polymers, and characterized according to molecular-weight cutoff (MWC), which refers to the average pore size created during the membrane manufacturing. A MWC of 10 kilodalton (kDa) is commonly used for dialysis of small molecular FH (11). For a molecule to effectively diffuse across a membrane, its molecular weight (MW) should be 20–50 times smaller than the MWC rating of the membrane. Various formats of dialysis devices are available; the most commonly used types of devices in methods intended for routine analysis of FHs are 96- or 48-well plate formats (94, 95).

Dialysis permits small molecules to pass through the membrane into the dialysis buffer, while large molecules remain in the sample compartment. After incubation (typically 6–24 h, depending on the design of the ED devices), an equilibrium between the sample and the buffer is established, and the buffer containing the FH of interest is analyzed to determine the FH concentration.

The observed concentration of FH after ED depends on (*a*) volume of the sample, (*b*) volume of the dialysis buffer, (*c*) the buffer composition, (*d*) size of the molecules being separated, material and MWC of the membrane, (*e*) incubation temperature, (*f*) agitation of the device during the dialysis process (e.g., rotation), (*g*) surface area of the membrane, and (*h*) adsorption of the FH of interest by the material of the membrane and the housing.

Potential pitfalls of ED are that (*a*) a membrane or seal may break during the pipetting, resulting in a contamination of the dialysate with a protein-bound analyte; (*b*) as FH is distributed between

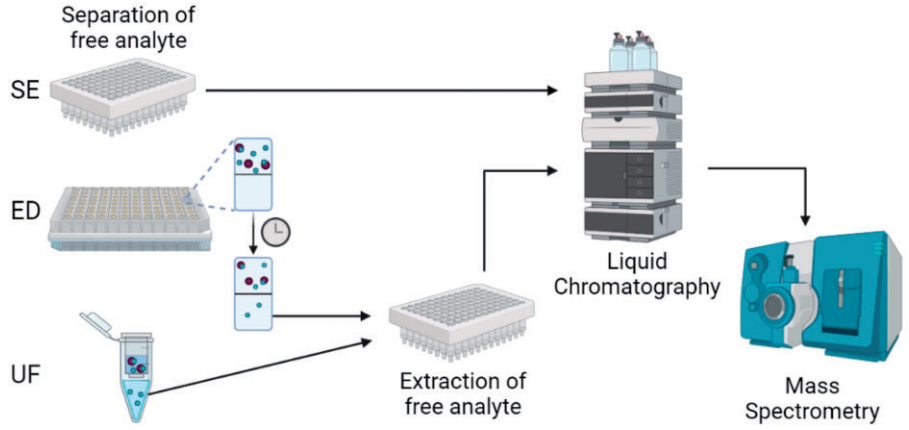

Fig. 1. Analysis of free hormone concentrations involves several basic steps. The first step of sample preparation involves separation of the free analyte of interest from protein-bound analyte. This can be done by size-exclusion (SE), equilibrium dialysis (ED), or ultrafiltration (UF). Next, internal standard (IS, stable isotope labeled analog of the FH of interest) is added to the samples, the isolated free analyte and the IS are extracted from the biological matrix, and the extracted free analyte and the IS are separated by liquid chromatography and detected by mass spectrometry. This figure was created with BioRender.com.

the 2 chambers, the equilibrium between protein-bound and FH is continuously reestablished (in the chamber containing biological samples) with new fractions of FH released from the carrier proteins to compensate for the FH migrated into the dialysis buffer; this may result in overestimation of the FH concentration; and (*c*) some hormones are stable in biological samples while bound to the carrier proteins and may get adsorbed by the surfaces when present in solution in a free form (in the dialysis buffer), resulting in underestimation of the FH concentration.

Ultrafiltration

UF is a process in which fluid passes through a semipermeable membrane due to an applied centrifugal force. Development of UF-based methods involve selection of the type of the UF device, with consideration of MWC, type of membrane material, housing and seal around the membrane, the ultrafiltrate collection container, and optimization of sample processing conditions (centrifugal force, temperature, and time of the centrifugation) (96).

UF membranes are like the MWC membranes used in ED devices but require larger MWC and should withstand pressure generated by the centrifugal force. A larger MWC pore size is required to avoid clogging of the membrane by large MW proteins that are present in biological samples.

A number of potential pitfalls could affect performance of UF-based devices and the observed concentrations of FH: (*a*) the full surface of the membrane is accessible for filtration only during the initial stages of the UF process; as filtration continues, the membrane gets clogged with high MW proteins significantly reducing the filtration rate and resulting in only a fraction of FH being filtered from the initial sample; (*b*) because every patient sample is unique, clogging may occur at different times during the UF process, resulting in variability in the volume of the filtrate and the resultant fraction of FH that is filtered; and (*c*) the centrifugation process generates heat, which

Table 4. Mass spectrometry-based methods for measurement of free hormones.

Analyte	Sample type	Sample volume, μL	LOQ	Method for separation FH. Temperature/time	Post-FH separation sample preparation	Number of analytes	Instrumental analysis	Chromatographic column	Inj-to-inj time, min	Ionization	Mass transitions, *m/z*	Reference interval	Reference
FTe	S, P	150	3 pg/mL	ED, 37 °C/20 h	DI, 2D LC	1	LC–MS/MS	First dimension: SecurityGuard C6 4 × 3 NR; second dimension: Synergy-Max RP, dimensions NR (Phenomenex)	4.5	APCI	*m/z* 289 to 97 and 289 to 109	24 age- and sex-specific reference intervals are provided	(24)
FTe	S	200	2.5 pg/mL	ED, 37 °C/time NR	LLE, DER	1	LC–MS/MS	Zorbax Extend-C18 2.1 × 50 mm 3.5 μm (Agilent)	5	ESI	*m/z* 289 to 97 and 289 to 109	Males: 34.5–173.8 pg/mL. Females: 2.5–6.4 pg/mL	(16)
FTe	S	200	5 pg/mL	ED, 37 °C/20 h	LLE, DER, 2D LC	1	LC–MS/MS	First dimension: SecurityGuard C6 4 × 3; second dimension: Kinetex C18 2.1 × 50 mm, 2.6 μm (Phenomenex)	4	ESI	*m/z* 304 to 112 and 304 to 124	Males 47–244 pg/mL	(25)
FTe	S, P	1000	0.6 pg/mL	UF, 37 °C/0.5 h	SPE/HPLC-fraction collection/DER	1	GC–MS	NR	NR; approximately 20	EI	*m/z* 680	NR	(16)
FTe	S, P	500	0.5 pg/μL	UF, NR/1 h	SLE, LLE, DER	1	LC–MS/MS	C-18 50 mm× 4.6 mm, 3 μm (Cadenza-CL, Imtakt)	5	ESI	*m/z* 403.3 to 164.2 and 403.3 to 152.2	NR	(90)
FTe	S, P	100	10 pg/mL	SES, 25 °C, 10 min	DER, DI	1	LC–MS/MS	First dimension: SecurityGuard C6 4 mm × 3 mm; second dimension: Kinetics C18 50 × 2.1, 2.6 μm (Phenomenex)	3	ESI	*m/z* 304.2 to 124.1 and 304.2 to 112.1	NR	(91)
FT4	S, P	600	2.5 pg/mL	UF, 25 °C, 1 h	DI	1	LC–MS/MS	LC-C18-DB (3.3 × 3.0 mm, 3 μm) (Supelco)	4	ESI	*m/z* 775.9 to 126.9	NR	(17)
FT4	S	First	2 pg/mL	ED, 37 °C/18 h	DI	1	LC–MS/MS	Kinetex C18 4.6 × 50 mm, 2.6 μm (Phenomenex)	NR	ESI	*m/z* 775.7/126.9	0.1–12 years: 8–14 ng/dL, 13–20 years 8–14 ng/dL,>20 years 08–18 ng/dL	(15)

Continued

Table 4. (continued)

Analyte	Sample type	Sample volume, µL	LOQ	Method for separation FH. Temperature/time	Post-FH separation sample preparation	Number of analytes	Instrumental analysis	Chromatographic column	Inj-to-inj time, min	Ionization	Mass transitions, *m/z*	Reference interval	Reference
FT4	S	NR	NR	ED, 37 °C/4 h	SPE, 2D LC	1	LC–MS/MS	First dimension: Acquity UPLC BEH300 C4; second dimension: Acquity UPLC BEH C18 (Waters)	NR	ESI	*m/z* 778 to 732	NR	(18)
FE2	S, P	250	0.05 pg/mL	ED, 37 °C/22 h	LLE, DER, 2D LC	1	LC–MS/MS	First dimension: C1 4 × 3 mm; second dimension: C6 phenyl 100 –2.1 mm 3 µm column (Phenomenex)	8	ESI	*m/z* 506.3 to 171.1 and *m/z* 506.3 to 156.1	Women by phase of menstrual cycle: <2.4 pg/mL follicular phase; <3.1 pg/mL mid-cycle; <2.6 pg/ml luteal phases	(22)
FPC	P	300	100 pg/mL	UF, 37 °C/0.5 h	DI, 2D LC	4	LC–MS/MS	Strata 20 × 2 cartridge (Phenomenex) and Zorbax-SB Phenyl, Rapid Resolution HT 100 × 2.1 mm 1.8 µm (Agilent)	11	APCI	*m/z* 363.1 to 327.2 and 363.1 to 121.1	NR	(19)
Free Serum Cortisol	S	NR	NR	ED, 37 °C/16 h	DI, 2D LC	1	LC–MS/MS	Oasis HLB (Waters) and Reprosil pur C18-AQ, 125 × 2 mm; 5 µm (Maisch)	NR	NR	*m/z* 363/309	NR	(20)
F25OHD2/D3	S, P	100	10 pg/mL	SES, 25 °C, 10 min	DER, DI	2	LC–MS/MS	First dimension: Zorbax Eclipse XDB-CN 50 × 2.1 mm 5 µm; second dimension: Poroshel SB-C18 50 × 3 2.7 µm (Agilent Technologies)	6	ESI	F25OHD3: *m/z* 732.5 to 673.5 and 732.5 to 217.2. F25OHD2: *m/z* 744.5 to 685.5 and 744.5 to 217.2	0.024–0.080 ng/mL	(91)

Serum (S), plasma (P), direct injection (DI), derivatization (DER), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), solid-phase extraction (SPE), liquid/liquid extraction (LLE), mass to change ratio (*m/z*), two-dimensional chromatographic separation (2D LC), equilibrium dialysis (ED), ultrafiltration (UF), size-exclusion separation (SES), injection-to-injection (Inj-to-inj), not reported (NR).

shifts equilibrium between the free- and the protein-bound hormone present in the processed samples. Use of temperature-controlled centrifuges partially mitigates the issue. However, during the initial stages of UF, while temperature of the samples is changing from ambient to the temperature that is intended for the separation, the filtration rate through the UF membrane is the highest and a large fraction of FH passes through the membrane before the temperature equilibrates.

FH concentrations measured by UF-based methods often do not agree with ED-based methods because of UF conditions (temperature, time, centrifugation speed), the type of the UF device (MWC membrane material, material of the of the housing, seal around the membrane, etc.), and inconsistencies in the filtration rate. Therefore, reference intervals are typically not interchangeable across methods for measurement of the same FH.

Size-Exclusion Separations

Size-exclusion separation (SES; also known as gel filtration chromatography) is another technique that may be used for separation of FH from biological samples. SES is a chromatographic separation technique utilizing columns containing a packed bed of polymeric beads, which have narrow distribution of the internal pore sizes. The underlying principle of SES is that molecules of different sizes move through the packed bed of SES beads at different rate. Molecules approximately <1/3 of the size of MWC of the pores, penetrate the pores and get retained. Molecules with MW above the MWC pass through the layer of the adsorbent, and molecules of intermediate size are partially retained by the adsorbent. Two important requirements for SES are that analyte functional groups should not interact with functional groups on surface of the adsorbent, and the separation should be controlled only by the size of the analyte molecules and the pore size distribution inside the particles of the adsorbent.

Traditionally, size-exclusion (SE)-based separations were performed using adsorbents packed in HPLC columns, with the separation using traditional HPLC instruments. Typical application of the SE chromatography is for separation of large molecules (e.g., glycans, proteins, polypeptides) (97). Research methods were developed for separation of protein- and peptide-hormones using SE-based online chromatographic separation (98–100). Because of the complexity of SES separations and relatively low efficiency of the SES using packed chromatographic columns, no online SES-based methods for small MW FH, intended for routine use, have been developed so far.

One recently introduced application of SES adsorbents is aimed on desalting samples (removal of salts and small molecules) from samples intended for analysis of proteins and peptides. The principle of the separations in these devices is based on the principle that small molecules may penetrate pores of the adsorbent and remain there, while molecules with MW exceeding the MWC of the SES adsorbent pass through the layer of the adsorbent. In these applications, the flow-through fraction is collected and used for LC–MS/MS analysis of large molecules.

In devices for offline SES, adsorbent is packed either in individual cartridges or in wells of 96-well solid-phase extraction (SPE) plates. Sample preparation steps for separation of FH using SES adsorbents are similar to the steps used in typical SPE methods using traditional for SPE adsorbents, with the exception that the sample and the solvents are forced through the packed bed of the adsorbent using centrifugation. A method for separation of free 25-hydroxy vitamins D2/D3 (F25OHD2/D3) and free testosterone (FTe) from biological samples using the offline SES devices was recently developed (86, 91, 101).

In methods for analysis of large molecules using SES devices for desalting, the fraction of interest is

the filtrate. In the method for separation of FH, the fraction of interest comprises small molecules, retained in the pores of the adsorbent, with the aim of depleting the sample from proteins, including carrier proteins containing the bound hormone of interest. In the developed method, sample loading is followed by washing the SES adsorbent and elution of the FH of interest from the adsorbent. For separation of FH from the biological samples, the sample is applied in the layer of adsorbent; while the sample resides in the interstitial space between the particles, FH diffuses into system of pores within the adsorbent and is retained in the pores.

The main difference between FH separation using SES adsorbents vs UF and ED devices, is that in ED and UF devices separation takes place on a surface of the porous membrane, while in the SES devices separation of FH occurs within a system of pores, having a thousands-of-times greater surface area compared to the linear surface of UF and ED membranes. The larger surface on which the separation takes place results in the greater efficiency and faster separation of FH from biological samples. Because SES adsorbents are chemically inert, separation is governed only by the ability of the sample constituents to penetrate the pores of the adsorbent. The hormone of interest, bound to the carrier protein, remains bound and elutes from the SES adsorbent along with other unretained proteins resulting in only FH that is present in the sample retained by the adsorbent. Offline-SES devices use centrifugation to force the sample through the adsorbent, washing the adsorbent, and elution of the FH of interest from the adsorbent.

In the developed method for FH separation using offline-SES devices, a biological sample is applied into the layer of an SES adsorbent where it is retained and allowed to equilibrate (91). During a brief incubation, FH diffuses from the retained biological sample in the interstitial space around the particles of the adsorbent into the pores of the adsorbent, while the high MW sample constituents remain in the interstitial space and are later removed from the adsorbent via centrifugation. FH separation is followed by wash of the adsorbent using aqueous solution, which removes the residual high MW sample constituents. After the washes, FH retained within the pores of the SES adsorbent, is eluted using organic solvent in which the FH is soluble. The eluting solvent is premixed with a stable isotope labeled analog of the FH of interest as an internal standard (IS). Following the elution, the solvent is evaporated, the residue is reconstituted, and the sample is analyzed on an instrument.

For methods in which sensitivity of detection is inadequate for measurement of endogenous concentrations of the FH of interest, a chemical derivatization could be used to enhance the sensitivity (see the section 'Sample preparation' that follows) (102).

These offline SES devices provide fast, high-efficiency separations, with the separated FH eluting in a small volume of solvent, enabling high sensitivity analyses. Use of offline SES devices for separation of FH from biological samples (91) provides a number of advantages over ED and UF including the following: (*a*) fast, within-minutes separation of FH from biological samples; (*b*) the fast separation prevents reestablishing equilibrium between the protein-bound hormone and the FH during the separation process, as occurs during the multihour ED separations, which can result in new portions of FH being released from the carrier protein while FH is transferred from the sample across the ED membrane into the dialysis buffer; (*c*) consistent performance over a wide range of protein concentrations in the biological samples (the system of pores within the particles does not get clogged as happens in the membrane of the UF devices); (*d*) ability to process samples in native conditions without disturbing the FH binding to their carrier proteins; (*e*) because of these issues, the observed FH

concentrations more accurately reflect the true FH concentration in the biological samples; (*f*) the devices enable separation of FH that are unstable in dialysis buffer (e.g., F25OHD2/D3) or tend to adsorb to the surfaces (86, 87, 91, 101); and (*g*) the offline-SES devices are available in a 96-well format allowing batch sample processing. Use of these devices was recently demonstrated for analysis of F25OHD2/D3 and FTe (86, 87, 91, 101).

All 3 described techniques for separation of FH (UF, ED, and offline SES) allow batch-type sample processing, with sample preparation decoupled from the instrumental analysis, enabling high-throughput routine testing of biological samples using mass spectrometry detection.

Extraction

After isolation of FH from biological samples, the resulting samples should be prepared for an instrumental analysis. This typically involves sample clean-up, concentration, and in some cases derivatization. Typical techniques used for extraction of small molecular FH are liquid–liquid extraction (LLE), and offline and online SPE (103–106). The extraction process facilitates reducing sample complexity, exchanging the sample solvent, and reducing matrix effects, thereby enabling greater sensitivity and standardized testing of samples for diagnostic applications.

LLE is based on acid–base equilibrium of protonated and deprotonated forms of analyte in solution and partitioning of analytes between aqueous sample and organic solvent, which is immiscible with the sample matrix. During LLE, polar solutes preferentially remain in the aqueous sample, while less polar solutes dissolve in the organic solvent. LLE is a simple, inexpensive, and practical approach for the extraction of small molecular FH (Table 4).

Polymeric and silica-based adsorbents have been used as SPE packing materials to extract FH from dialysates and ultrafiltrates (107). Benefits of SPE include reduction of sample complexity, concentration of the sample (enhancing analytical sensitivity), and reduction of impurities introduced in the instrument during the analysis.

SPE is performed by passing the sample through cartridges containing packed SPE adsorbent using vacuum or positive pressure. After separation of FH from a biological sample using ED, UF, or SES techniques, the samples are applied to SPE cartridges; the FH of interest is retained by the adsorbent while many of the sample constituents either pass through adsorbent or are weakly retained and removed during the washes performed before elution of the analyte of interest. Following elution, the solvent is evaporated, the residue is reconstituted in a small volume of a solvent compatible with the LC separation, and the sample is analyzed (107). The main advantage of SPE in comparison with LLE is in greater specificity. SPE can be performed offline, for a batch-type sample preparation, or online.

SPE and other extraction techniques for FHs of clinical interest typically do not recover the entire amount of the FH recovered from the biological specimen. While these losses affect the absolute FH recovery, in well-developed methods, the reduced recovery does not affect accuracy of the measurement. Variability in the recovery of FH during extraction and instrumental analysis is compensated by the recovery of IS, which correlates with recovery of the FH of interest. Therefore, typically only 20%–50% of the absolute amount of the FH is recovered from the biological sample and injected into the instrument.

Online SPE is a trap-and-elute technique, allowing sample clean-up before chromatographic separation. After separation of FH, the samples are free of large molecules (proteins, etc.) and are usually compatible with direct injection using the online SPE strategy for analysis.

Online SPE involves coupling the separation of the analyte of interest on a reusable SPE cartridge (first dimension separation) with separation on a

chromatographic column (second dimension separation). Online SPE is often used with tandem mass spectrometry (MS/MS) detection, and is shown to be a powerful technique allowing direct injection of dialysates and ultrafiltrates for FH analysis (21). Online SPE methods require use of 2 pumps and a switching valve. Analysis consists of injecting dialysate or ultrafiltrate premixed with an IS in the reusable SPE cartridge; effluent from the cartridge is directed to waste, while the FH of interest is retained in the adsorbent of the cartridge. After the cartridge is cleaned with a weak solvent, the switching valve is rotated to connect the cartridge and the analytical column in series. The analyte is transferred from the cartridge to the analytical column, chromatographically separated on the analytical column, and effluent from the analytical column is directed into an MS/MS ion source (21, 108, 109).

Advantages of online SPE include (*a*) the reduction of sample handling; (*b*) reduced potential for adsorptive losses and incomplete recovery of FH; (*c*) the possibility of high volume sample injection; (*d*) greater analytical sensitivity (the entire sample obtained after separation of the FH can be injected for instrumental analysis, while methods using offline sample preparation, only a fraction of the final sample can be injected); and (*d*) simplified sample preparation leads to reduction of potential adsorptive losses, and reduces cost and labor.

Methods using online SPE typically have comparable accuracy and precision along with greater sensitivity and reduced time for sample preparation (21, 25, 109) compared to traditional extraction techniques. Methods for FT3, FT4, FTe, and FE2 that use online SPE have been developed and routinely used in clinical laboratories (21, 25, 26, 109).

A variation of the online SPE approach is two-dimensional chromatographic separation (2D-LC); such methods may be useful in cases where separation of FH from interfering peaks or reduction in potential for ion suppression are needed. 2D-LC uses a similar instrument setup to online SPE, however, instead of an SPE cartridge, the first dimension separation is performed on a chromatographic column. Using this approach, only a small fraction of elution from the first dimension separation is transferred to the analytical HPLC column. The 2D-LC separations have several advantages, include the following: (*a*) high-efficiency separation on the first dimension results in a small fraction of the elution from the first dimension separation transferred in the analytical column; (*b*) enhanced resolving power afforded by different selectivity of the first dimension and the second dimension HPLC columns; and (*c*) because the first dimension separation removes a large fraction of the matrix impurities (e.g., salts, early and late-eluting peaks) to waste, there is typically lower background noise, greater analytical sensitivity, lower potential for ion suppression, and reduced contamination of the mass analyzer.

Analyte derivatization is a technique that can be used for enhancement of sensitivity and specificity in measurements of FH using LC–MS/MS (25, 102). Derivatization increases the MW of the molecules, resulting in a change in the mass-to-charge ratios (*m/z*) of the molecular and fragment ions, compared to underivatized molecules. Derivatization changes the chemical properties of the molecules, including ionization efficiency, volatility, fragmentation, chromatographic retention, and stability (25, 26, 86). Most derivatization techniques used for electrospray ionization (ESI) incorporate permanently charged or ionizable functional groups, whereas derivatization used with atmospheric pressure chemical ionization typically involves incorporation of functional groups with high proton or electron affinity (25, 26, 86, 90, 91).

Functional groups commonly targeted for derivatization of endogenous steroid hormones are keto, hydroxyl, and diene groups (25, 26, 91). Derivatization was successfully used in methods for analysis of FTe (25), FE2 (22), and F25OHD2/D3

(86, 87, 91). Derivatization can be beneficial in cases when the required sensitivity cannot be achieved with conventional techniques and when smaller sample aliquot is beneficial (e.g., pediatric testing). It should be noted that in some cases, derivatization increases complexity of sample preparation, and sample clean-up from the derivatization reaction byproducts could be required.

There is no single extraction technique that can be applied to measurement of all FHs. Selection of a method for separation of FH from biological samples and instrumental analysis depends on the chemical properties of the analyte of interest, the stability of the FH in solution after separation from biological samples, the presence of isobaric interfering substances, and the required sensitivity. Mass spectrometry-based methods are not free from the possibility of interference derived in vivo or during the preanalytical phase. For instance, in vivo generation of nonesterified fatty acids due to heparin treatment can shift the TH/FH equilibrium in vitro during the ED incubation (6, 60).

Since the first mass spectrometry-based methods for FH analysis were developed and introduced in clinical practice, significant advances have been made in mass spectrometry sensitivity, chromatography instrument capabilities, and consumables resulting in a significant enhancement in the achievable sensitivity in FH analysis. These technological advances could allow for FH measurement in individuals in which endogenous concentrations are too low for detection using earlier available instrumentation and methods (e.g., FTe in children and women, FE2 in children and men), or in cases where analyte derivatization was necessary (e.g., free testosterone in men, FE2 in women of reproductive age).

LC–MS/MS Analysis

Chromatographic separation of free steroid hormones is commonly performed on reversed phase (C18) HPLC columns (Table 4). During the last 10 years, ultra-HPLC with sub-2 μm and core-shell particle columns have been used. These separations have higher efficiency, especially when coupled with mass spectrometers capable of high-speed data acquisition, and offer advantages of enhanced resolution, sensitivity, and speed of analysis.

The most common ionization techniques used for LC–MS are ESI and atmospheric pressure chemical ionization (110, 111), and a triple-quadrupole mass spectrometer (MS/MS) is the main type of mass analyzer used for quantitative analysis of FH (111). Advantages of MS/MS compared to other types of mass analyzers include greater sensitivity and specificity. The most sensitive MS/MS mode of acquisition for quantitative analysis is multiple-reaction monitoring (MRM) (111, 112), due to the ability of the mass analyzer to continuously monitor a predefined set of *m/z* during the data acquisition. Specificity is assured by monitoring multiple (typically 2) FH-specific and IS-specific mass transitions. Well-developed and validated MS/MS-based methods (113) are more specific than most other analytical techniques. One advantage of MS/MS-based methods is the ability to assess specificity of analysis and analyte recovery in every sample. This can be accomplished through monitoring multiple mass transitions of the analyte and the IS, and the peak intensity of the IS (113, 114).

Compounds that may potentially interfere with MS/MS methods include isobars (molecular species with the same nominal mass but different exact mass) of the target analyte [e.g., dehydroepiandrosterone (DHEA) with testosterone (Te)] and adducts or isotopic ions of the impurities, which are isobaric to the analyte of interest or the IS (115). Common types of interference among steroids are isomers and A + 2 isotopes of their unsaturated analogs [e.g., cortisone with cortisol; estrone with estradiol (E2); prednisolone with cortisol]. Interference in methods using MS/MS

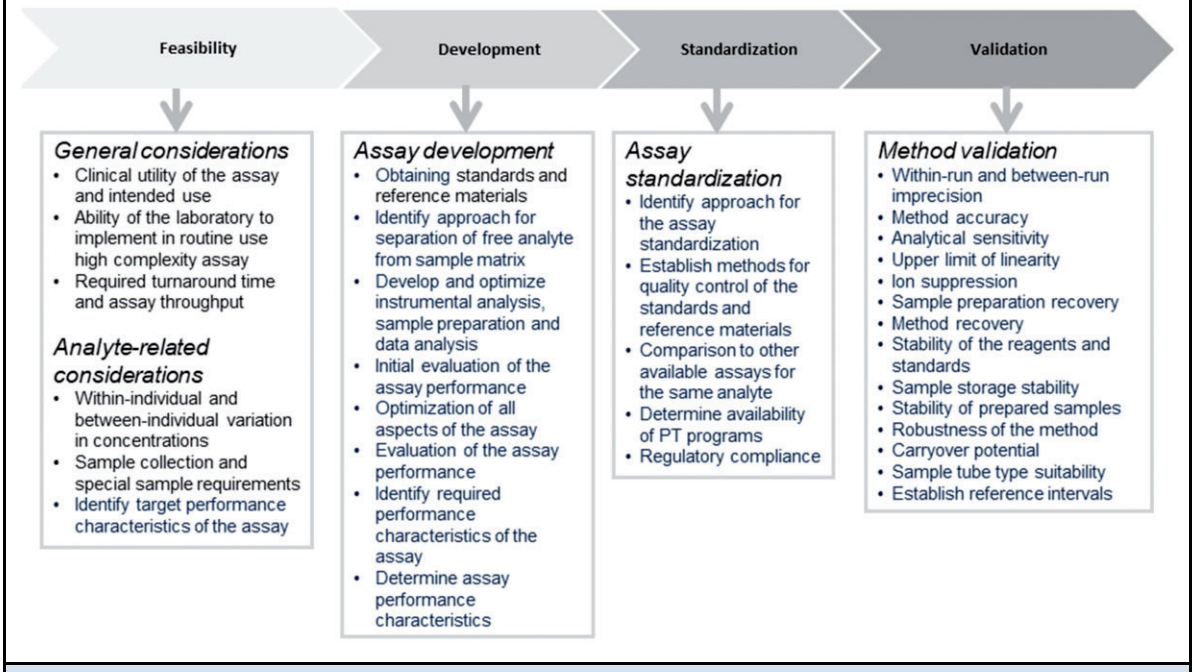

Fig. 2. Workflow for development and validation of laboratory developed tests for analysis of free hormones.

detection also may be caused by conjugates of the analytes of interest (e.g., glucuronide, sulfate). Exposure of the conjugates to elevated temperatures in the ion source could result in their hydrolysis and formation of free analyte, which would falsely elevate measured concentrations of FH; this may occur in methods that do not chromatographically resolve peaks of the targeted FH and the FH's conjugates.

Mass transitions used in MS/MS methods should be evaluated to assure that only the targeted analyte is measured and that the method does not suffer from interferences (114). Evaluation of interference potential must include analysis of isomers and isobars of the target analyte, compounds structurally related to the analyte, common endogenous sample constituents, drugs that might be administered to the targeted population, commonly prescribed drugs, or drug metabolites (114, 115). Ion suppression is another type of interference that may affect the specificity of MS/MS-based methods. Multiple approaches have been developed to assess ion suppression in analytical methods (116). Analyte-specific extraction and use of stable isotope labeled standards are general approaches used to minimize matrix effects and to improve analytical accuracy.

Laboratory Developed Tests for FH, Reference Intervals, and Standardization

A guiding document related to development, validation, standardization, and implementation of commercially manufactured assays for FT4 was published in 2004 (11), while there are no guidelines available so far that are related to development of laboratory developed tests (LDT) for physiologically important FHs.

Figure 2 summarizes considerations for developing mass spectrometry-based LDTs for FH, including assessment of feasibility, as well as

aspects of method development and validation. The Clinical and Laboratory Standards Institute (CLSI) published recommendations on clinical mass spectrometry, i.e., documents C50-A, C57, and C62-A, which should serve as a guideline for development and validation of the clinical mass spectrometry-based methods (113, 117, 118).

To interpret FH test results, it is necessary to establish reference intervals as detailed in CLSI guideline EP28-A3c (119). Population-based reference intervals are usually determined for well-defined groups of individuals, which are like the targeted population (sex, age distribution, etc.) in all aspects, except absence of diseases (or medications), influencing FH concentrations. Typically, nonparametric, and robust statistical methods are preferred for establishing reference intervals of clinical biomarkers, because they are not based on assumptions of the probability distribution. On rare occasions when biomarker concentrations in the reference populations are normally distributed, parametric methods can be used.

Well-developed and validated mass spectrometry-based methods are typically more specific, compared to other techniques. Because of this, reference intervals should be established for each FH method before they are introduced in routine clinical use. Thorough method validation, standardization, and harmonization of mass spectrometry-based methods for FHs among different laboratories, should facilitate the implementation of uniform reference intervals for LC–MS/MS-based methods for FHs (113).

Nonharmonized FH reference methods and unique patient demographics require that each laboratory establishes pertinent reference intervals. Patient care could be improved, however, if methods for FHs would be harmonized across laboratories, allowing for adoption of reference intervals among laboratories. In addition to method-related differences, there are among-population and -ethnicity differences in the distributions of the FH concentrations characteristic of health, which necessitate development of population- and ethnicity-specific reference intervals for FHs.

Last, lot-to-lot variability in consumables (ED, UF, or SES devices) used for isolation of FHs, and among-method or among-procedure differences for FH separation from biological samples, may contribute to the greater assay variability compared to methods for measurement of TH concentration.

CONCLUSION

In vitro measurements of FHs provide a glimpse into the dynamic hormone equilibrium in vivo: that is, continually adapting to acute physiologic demands or disturbances due to a particular physiologic state, a binding protein abnormality, or medication use. However, FH measurements correlate with physiological hormone effects and are a standard clinical tool for the assessment of thyroxine, sex hormone, and cortisol status.

Direct methods that isolate FH, followed by LC–MS/MS analysis, are available at many clinical reference laboratories for measurements of FTe, FE2, FT4, FT3, and free plasma cortisol. As shown in many studies, these methods provide specific analytical advantage over commercial immunoassays and indirect (e.g., calculation-based) methods. High-throughput methods for physiologically important FHs have been developed and used for routine patient sample testing, providing adequate turnaround time and cost efficiency. Mass spectrometry-based methods are considered a gold standard method (provide higher analytical sensitivity and specificity compared to other available techniques) for measurement of FH concentrations. Multiple barriers currently prevent more widespread use of direct reference methods for measurement of FHs, including technical complexities that are best suited for clinical reference

laboratories. Furthermore, sample preparation for direct measurement of FHs is time consuming, with ED methods often requiring overnight incubation, thereby increasing assay turnaround time. Emerging techniques such as SES, as well as current efforts to harmonize and standardize FH assays to make them more reliable, may alleviate the challenges of assay reliability and allow for reduced turnaround time. Laboratories with experience in high-complexity workflows and quality programs in place may be best suited for implementing methods for direct measurement of FH using LC–MS/MS. Laboratories should provide adequate information in directories to appropriately highlight the clinical conditions, advantages, and limitations of FH assays to promote clinical education and guidance regarding the appropriate use of FH measurements. As research continues to reveal clinical associations between binding protein abnormalities and hormone physiology, it is likely that mass spectrometry-based methods for direct measurement of FH concentrations will be more widely used in future clinical practice.

Nonstandard Abbreviations: FH, free hormone; TH, total hormone; ED, equilibrium dialysis; UF, ultrafiltration; SES, size-exclusion separations; MWC, molecular-weight cutoff; MW, molecular weight; SE, size exclusion; SPE, solid-phase extraction; FTe, free testosterone; F25OHD2/D3, free 25-hydroxy vitamins D2/D3; IS, internal standard; LLE, liquid/liquid extraction; FT3, free triiodothyronine; FT4, free thyroxine; FE2, free estradiol; 2D-LC, two-dimensional chromatographic separation; *m/z*, mass-to-charge ratio; ESI, electrospray ionization

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