serum for the diagnosis of thyroid disease; it is the unbound, biologically active hormone component in serum as implied by the free-hormone concept. However, the accurate measurement of free thyroid hormones presents a challenge since the vast majority of thyroid hormone (>99.5%) is protein bound, with the remaining free fraction present at picomolar levels, necessitating highly sensitive assays for detection and quantification of free hormone.

The procedure of equilibrium dialysis is considered the 'gold standard' for free-hormone analysis and constitutes a preliminary separation step by use of a semipermeable membrane to physically separate the free fraction from the protein-bound fraction prior to free-hormone quantification. Until recently, this method has been considered not practicable for routine free-hormone measurement, and its use has been confined to specialist laboratories. However, modern equilibrium dialysis methods are now becoming available that employ high surface-to-volume ratio to reduce the time taken to attain equilibrium and are automation compatible to improve efficiency, thus having potential for future routine application.

The concept of free-hormone assays for T3 and T4, due to the low molecular weight of these target analytes, are based on the competitive immunoassay format which, critically, is designed to conserve the equilibrium between free and protein-bound hormone in serum during the assay process, to ensure that the assay signal generated reflects the 'free' rather than the 'total' hormone concentration. Commercial free-hormone assays for thyroid hormone measurement are based on either a 'one-step analogue' or a 'two-step' immunoassay format. In the one-step assay, a labelled T4 (or T3) analogue is employed which has binding affinity for the reagent antibody but, critically, has minimal affinity for endogenous serum-binding proteins. During the assay reaction, labelled analogue tracer competes for antibody-binding sites with endogenous hormone, and the subsequent assay signal generated is inversely proportional to the serum free-hormone concentration. The two-step format in contrast employs a separation step whereby a small quantity of reagent antibody is first incubated with serum to bind free hormone with minimal perturbation in the serum-free and protein-bound hormone equilibrium. This incubation is followed by a wash step to remove serum from the immobilized antibody, and then subsequent addition of tracer for measurement of free-hormone concentration.

Assays for free-hormone measurement are potentially confounded by factors that affect the serum free-to-bound hormone equilibrium. These factors include the heparin-mediated activation of endothelial lipoprotein lipase; this activation results in the production of free fatty acids that displace albumin-bound thyroid hormone. Additionally, genetic variants of binding proteins such as observed in familial dysalbuminaemic hyperthyroxinaemia (FDH), in which mutations of the albumin gene increase the affinity of albumin for T4 by approximately 60-fold, leading to overestimation of the FT4 concentration (particularly when measured by one-step analogue assays). In suspected cases of FDH, measurement of FT4 by equilibrium dialysis is appropriate for the elimination of FT4 assay interference.

Immunoassay in practice

Immunoassay methods are readily adaptable to automated analysers and subsequently require limited technical input by laboratory staff. In addition, automation enables rapid throughput and turnaround of laboratory test results, making automated immunoassays ideally suited to routine hormone measurement. However, immunoassays have a number of potential analytical pitfalls, including an inherent vulnerability to interference from anti-reagent antibodies and endogenous autoantibodies, susceptibility to cross-reactivity with structurally related compounds, macro-complex interference, and the high-dose hook effect (Table 1.1). These susceptibilities may give rise to false-positive or false-negative results, some examples of which are now briefly discussed. Interference in immunoassays due to anti-reagent antibodies can be categorized by the type of interfering antibody: (1) human anti-mouse antibodies (HAMAs), which are produced by the immune system in response to a direct antigenic stimulus and are sometimes observed in patients who work closely with animals, or in patients who have received therapeutic mouse monoclonal antibodies for imaging or treatment purposes; (2) heterophilic antibodies that, in contrast to HAMAs, are polyclonal and have variable affinities for antibody epitopes, so that immunoassay interference by heterophiles is generally less pronounced than that caused by HAMAs; and (3) rheumatoid factor autoantibodies that bind to the Fc portion of IgG, resulting in interference in the immunoassay and which are often found in the sera of patients with rheumatoid conditions.

Interference in thyroglobulin immunoassay from endogenous thyroglobulin autoantibodies can be particularly problematic, as such autoantibodies are often present in patients with autoimmune thyroid disease and differentiated thyroid carcinoma. Depending on whether the thyroglobulin–autoantibody complex partitions into the free or bound immunoassay fraction, the assay will generate a spuriously high or low result. Such interference has obvious clinical implications, since an undetectable thyroglobulin result after TSH stimulation is a marker of remission of differentiated thyroid carcinoma.

The specificity of reagent antibodies in an immunoassay formulation will determine the assay's vulnerability to structurally related compounds that harbour common cross-reactive epitopes with the analyte of interest. Cross-reacting substances may be (1) an endogenous compound with close structural homology with the target analyte (e.g. human chorionic gonadotrophin (hCG) cross-reactivity in a luteinizing hormone (LH) assay (both hCG and LH having a common alpha subunit)); (2) an endogenous compound that is a precursor in a metabolic pathway (e.g. in patients receiving the 11-beta-hydroxylase inhibitor metyrapone for the medical management of Cushing's syndrome, circulating levels of the cortisol precursor 11-deoxycortisol may accumulate, potentially leading to subsequent unrecognized hypoadrenalism due to spuriously elevated cortisol immunoassay results as a consequence of 11-deoxycortisol cross-reactivity); or (3) an exogenous medication (e.g. cross-reactivity of the growth hormone receptor antagonist pegvisomant in a growth hormone assay; insulin analogues that may cross-react in an insulin assay; and prednisolone cross-reactivity in a cortisol assay).

Macromolecular complexes, as exemplified by macroprolactin, which comprises prolactin bound to an anti-prolactin IgG autoantibody, give rise to hyperprolactinaemia in the absence of pituitary disease and may lead to medical mismanagement of

Table 1.1 Potential analytical interferences in immunoassays

Interference	Mechanism	Example
Cross-reactivity	Structurally-related compounds in the specimen are detected by the IA and generate a false test result.	- Prednisolone cross-reactivity in cortisol assay. - hCG cross-reactivity in LH assay
Anti-reagent antibodies	*Anti-reagent antibodies bind to the capture and/or detection Ab in the IA causing false test result.	- Human anti-mouse antibodies (HAMA) - Heterophilic antibodies - Rheumatoid factor
Autoantibodies	Autoantibody-analyte complex formation causing false test result.	Immunoassays that may be affected by autoantibodies include thyroglobulin, insulin and thyroid hormone assays.
Hereditary binding protein abnormalities	Genetic variants of binding proteins that may cause false test results.	**Albumin variant in FDH has altered binding affinity for T4 causing elevated FT4 result.
Macro-complexes	Immunoglobulin-analyte complex causing a falsely elevated test result.	- Macroprolactin is the most well documented example. Other reported complexes include: - Macro-TSH - Macro-CK (a macroenzyme).
High-dose hook effect	At very high analyte concentration, capture and detection Ab in two-site immunometric assays become saturated, inhibiting sandwich complex formation causing a falsely low test result.	Potential to affect compounds that circulate at very high concentration in the pathophysiological state, including hCG, prolactin and thyroglobulin.

Abbreviations: Ab, antibody; FDH, familial dysalbuminaemic hyperthyroxinaemia; FT4, free thyroxine; hCG, human chorionic gonadotrophin; IA, immunoassay; LH, luteinizing hormone; macro-CK, macro-creatine kinase; macro-TSH, macro-thyroid-stimulating hormone; T4, thyroxine.

*More likely to affect two-site immunometric assay format.

**More likely to affect one-step analogue assays.

patients. Prolactin circulates predominantly as a monomeric 23 kDa form; however, the high molecular weight macroprolactin form may have significant immunoreactivity in conventional prolactin immunoassays yet is minimally bioactive in vivo. It is pertinent to note that macroprolactin may be present in patients with elevated monomeric prolactin, and screening for macroprolactinaemia above an agreed serum prolactin cut-off concentration is widely practised by clinical laboratories. Additionally, prolactin is by no means the only hormone for which macromolecular phenomena have been described; isolated elevations in TSH may, in very rare cases, be caused by macro-TSH that can confound the interpretation of thyroid function test results in the absence of thyroid symptoms.

The high-dose hook effect (sometimes referred to as the pro-zone effect) occurs when analyte is present at very high concentration, leading to the saturation of reagent antibodies in an immunoassay. This is observed in two-site immunometric assays in which the capture and detection antibodies are added to the reaction simultaneously, and the risk is obviously greatest for hormones that have a wide (patho)physiological concentration range, such as hCG, prolactin, and thyroglobulin. Indeed, the effect may be so great that results hook back to within the normal reference range.

Clinical laboratories utilize a variety of testing strategies to investigate possible interference in immunoassays but communication between clinical and laboratory staff is vital when results are incongruent with the clinical picture. Ultimately, minimizing the risk of interference in immunoassays is a shared responsibility. A thorough description of immunoassay interference is beyond the scope of this chapter and the reader is referred to comprehensive reviews in this area for further reading.

Mass spectrometry

Since the turn of the millennium, liquid chromatography–tandem mass spectrometry has become increasingly prominent in clinical laboratories for the accurate quantification of low-molecular-weight molecules, such as endogenous metabolites, drugs for therapeutic monitoring, and, in the context of endocrinology, steroid hormones. This technique commands exquisite analytical sensitivity and specificity by virtue of its ability to detect and discriminate ions based on mass:charge ratio (m/z), acting as a 'molecular sieve' to filter and quantify the analyte of interest. The dominant detector configuration in current clinical diagnostic applications is the tandem mass spectrometer. Liquid chromatography is used at the front end of the instrument to separate compounds and thus minimize matrix effects and isobaric interferences. The subsequent column eluent undergoes ionization to form charged ions (e.g. m/z of a singly charged ion $[M + H]^+$ = molecular weight +1) before infusion into the ion source of the mass spectrometer. This tandem configuration permits the selection of a precursor ion (parent ion) for stable trajectory through the first quadrupole (MS1), where it is directed into the second quadrupole (the collision cell), where it undergoes fragmentation via collision-induced dissociation. The resulting ion fragments, which are indicative of the structure of the parent molecule enter the third quadrupole (MS2), which is tuned to permit a selected product ion (i.e. the daughter ion) to traverse it for quantification at the detector (see Figure 1.4).

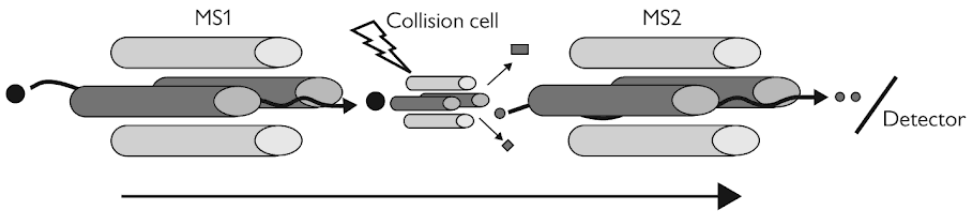

Fig 1.4 Tandem mass spectrometry; the parent ion passes through the first quadrupole (MS1), where it is directed into the second quadrupole (the collision cell), where it undergoes fragmentation via collision-induced dissociation. The resulting ion fragments that are indicative of the structure of the parent molecule enter the third quadrupole (MS2), which is tuned to permit a selected product ion (i.e. the daughter ion) to traverse it for quantification at the detector.

Mass spectrometry in practice

The ability of mass spectrometry to identify and quantify analytes based on their molecular weight offers enhanced analytical specificity. Additionally, recent advances in both mass spectrometry and online sample preparation technology (pre-analytics) has progressed the analytical sensitivity of this methodology to the extent that modern mass spectrometry applications are now comparable to conventional immunoassay methods in this regard. Additionally, mass spectrometry is now entering the arena of peptide quantification, exemplified by the availability of mass spectrometry-based assays for plasma renin activity by measurement of the peptide angiotensin I. Furthermore, protein quantification by mass spectrometry is now possible, with the recent documentation of mass spectrometry assays for insulin-like growth factor 1.

Like immunoassays, mass spectrometry is not impervious to the potential for analytical error. For example, mass spectrometry applications in clinical diagnostic laboratories are generally developed 'in house' and, consequently, mass spectrometry methods for the same target analyte may differ significantly in their application protocols, with potential variables including chromatography strategy, ionization process, mass transitions employed, and the use of different internal standards. As such, differing mass spectrometry method protocols may introduce analytical bias between different laboratories. The potential sources of interference in mass spectrometry are as follows:

- ionization: the presence of compounds in the sample that affect the efficiency of the ionization process prior to starting mass spectrometry; such matrix effects cause ion suppression/enhancement which may differentially impact the target analyte and internal standard, resulting in inaccurate test results
- internal standard: deuterated internal standard may have the same mass as a naturally occurring isotope within the mass distribution of the target analyte; thus, the naturally occurring isotope will increase the amount of internal standard detected, therefore decreasing the relative response for the target analyte and causing the analyte concentration to be underestimated
- mass transition selection: isobaric compounds that share the same m/z as the target analyte may lead to incorrect test results; chromatography should be optimized to eliminate this type of interference

Mass spectrometry methods for clinical applications must be rigorously validated prior to implementation into clinical care, and potential sources of bias must be ascertained and systematically addressed. Mass spectrometry is a sequential analytical technique and, consequently, analysis times are generally longer in comparison to automated high-throughput multichannel immunoassay methods. However, for medium-to-high-throughput laboratories, mass spectrometry offers the potential for operational cost savings.

Conclusion

It is important for clinicians to have an understanding of the principles and pitfalls of hormone measurement. This knowledge is a great asset towards the appropriate requesting and interpretation of hormone tests.

There is currently great debate in the biochemical endocrinology community regarding the appropriate use of assays and, indeed, which methodology is best suited to the diagnostic laboratory. The decision as to whether to use immunoassays or mass spectrometry has polarized opinion; advocates of immunoassays have extolled the ease of use and high sample throughput that automated immunoassay platforms offer, whilst mass spectrometrists have praised the superior analytical robustness of mass spectrometry, which lends excellent analytical sensitivity and specificity with minimal susceptibility to assay interference. The truth is that both of these techniques offer advantages for the clinical laboratory, and the individual strengths of each method can be complimentary when applied in the appropriate manner for endocrine investigations.

Further reading

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1.3 Hormone measurements: Hormone-binding proteins

Introduction

Most hormones circulate in blood extensively bound to proteins. This protein binding results in only a small fraction of the hormone being unbound and available to have biological effects and therefore modulates hormone bioavailability. The amount of protein-bound hormone is variable, but generally hormone assays measure the total (i.e. bound and unbound) hormone concentration in the circulation. Therefore, protein binding is an important determinant, and often caveat, in hormone measurement, as it defines what exactly is measured and how. In this section, known physiological and pathophysiological roles of hormone-binding proteins are briefly described. The significance of protein binding in hormone measurement is then highlighted. Although most hormones have a protein-binding system, the stress of this section is on the binding proteins that are most studied. More specifically, corticosteroid-binding globulin (CBG) is used as the main example, as it typifies the role of most specific hormone-binding proteins.

Carrier proteins and hormone reservoirs

Steroid and thyroid hormones are hydrophobic molecules that require a transfer system in plasma in order to remain soluble, circulate, and reach their target tissues. Hormone-binding proteins can be divided into hormone-specific systems, which usually bind hormones with high affinity and low capacity, and non-hormone-specific binding proteins, which bind with low affinity but high capacity due to their much higher concentrations in serum. The major non-hormone-specific binding protein is albumin, which, along with transthyretin, binds lipophilic hormones. The most extensively studied major hormone-specific binding proteins are CBG, sex hormone-binding globulin (SHBG), and thyroxine-binding globulin (TBG). Although specific binding proteins have been described for other hormones, such as growth hormone and prolactin, here we will use CBG as an example, since the physiological role of CBG is better understood.

CBG is the main plasma transporter for glucocorticoids and binds up to 90% of cortisol. Furthermore, various other endogenous steroids bind to CBG with lower affinity, including testosterone and progesterone. The binding of exogenous glucocorticoids is variable and, although some bind significantly, like prednisolone, others, such as dexamethasone, do not. Critically, CBG acts as a circulating steroid buffer, protecting tissues from steep oscillations in adrenal production of cortisol. The amount of cortisol bound to CBG defines the bioavailable fraction and acts as a readily releasable steroid reservoir.

Modulators of hormone action

CBG is a typical example of a hormone-binding protein that has an active role in modulating hormone action. Detailed structural and functional studies of CBG have revealed a biological role that extends beyond that expected from a simple carrier molecule. As a member of the serine proteinase inhibitor (serpin) family of proteins, CBG undergoes a defined conformational change upon interaction with target proteinases, and this change influences its functionality as a steroid-binding protein. More specifically, when human CBG is cleaved by neutrophil elastase, this causes a structural rearrangement and substantial loss of cortisol-binding activity, providing a mechanism by which CBG regulates the local delivery of cortisol to target tissues during inflammation. Moreover, CBG behaves as a negative acute phase protein during acute inflammation. Interleukin 6 directly inhibits CBG gene transcription and protein secretion, and exogenous interleukin 6 administration decreases serum CBG concentration by 50%, with levels normalizing after 7 days following a single injection. As a result of reduced hepatic synthesis and increased degradation by elastase, substantial decreases in plasma CBG levels occur in patients with systemic infections, sepsis, severe burns, and myocardial infarction, and this likely ensures that end organs receive a maximal supply of glucocorticoids at multiple levels to control inflammation, gluconeogenesis, and stress.

Significance in hormone measurement

The unbound hormone fraction defines the biological hormone action and, therefore, the feedback regulation of hormone production. As an example, when protein binding is increased, the unbound moiety transiently decreases and this causes increased production of the hormone, due to positive feedback regulation of the hormone axis. This results in a rise in the total circulating hormone concentration, whereas the opposite is observed for reduced protein binding. These changes in CBG and total serum cortisol concentrations are not associated with a phenotypical change, as the unbound bioavailable cortisol remains unaltered. The concentration of binding proteins is regulated by several factors that control production, secretion, and clearance, and this section refers to the most common factors encountered in clinical practice.

CBG has a long half-life of 5–6 days, and factors that modulate secretion but not clearance only affect circulating CBG levels after prolonged exposure. Among the factors that have a clinically relevant effect on CBG and total serum cortisol concentration (Table 1.2), the oestrogen-related increase in CBG is probably the most well documented, contributing to the observed increase of measured total serum cortisol in women treated with oral contraceptives or who are pregnant. Pregnancy-associated CBG glycoforms with increased sialic acid groups have also been identified, possibly resulting in slower clearance. Mitotane, an adrenolytic agent used in the management of adrenal cancer, has oestrogenic properties and causes an increase in total serum cortisol; this increase has to be accounted for in treatment monitoring. In a similar manner, alterations in SHBG concentration affect the total testosterone concentration without changing the corresponding free or bioavailable testosterone concentration. Conditions that alter circulating SHBG concentration are shown in Table 1.3. When protein binding is altered, it is generally accepted that the unbound hormone fraction is not affected and retains normal pharmacokinetics; therefore, this should influence the choice of hormone measurement. This is described in more detail in Section 1.4. Genetic variations in binding-protein concentration and/or binding affinity also impact on total hormone concentration to a clinically important extent.