Hormone Assays

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Hormones

- Hormones are the member of a class of signaling molecules produced by glands in multicellular organism that are transported by the circulatory system to target distant organ to regulate physiology and behavior.

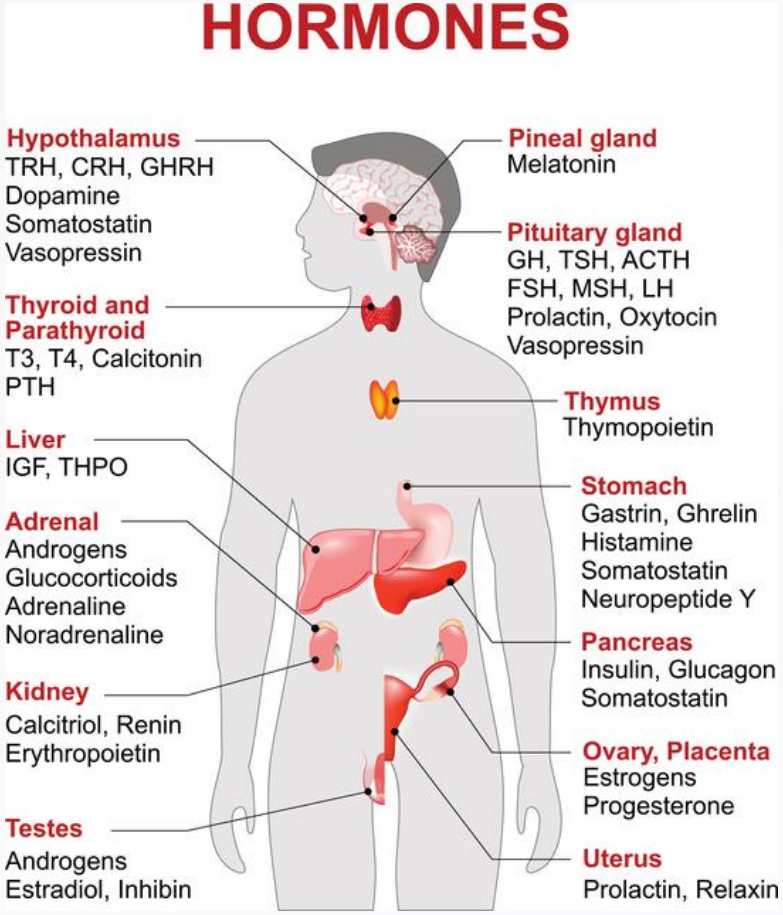
- Hormones are regarded as chemical messengers, communicating between organs and tissue for physiological regulation and behavioral activities.

Classification of Hormones

- Broadly classified in to two categories:

1) According to Chemical Nature

2) On the basis of Mechanism of Action



- **According to Chemical Nature**

A) Steriod Hormones :

i) Corticosteriods : From Adrenal cortex: Mineralocorticoids & Glucocorticoids
ii) Sex Steriods : Testosterone, Estrogen, Progesterone

B) Amine Hormones : Derived from amino acid tyrosine

i) Thyroid : T3 & T4
ii) Adrenal Medulla : Catecholamine (Adrenaline, Nor-adrenaline)

C) Peptide Hormones : Insulin, Glucagon, Oxytocin

D) Glycoprotein Hormones : FSH, LH, TSH

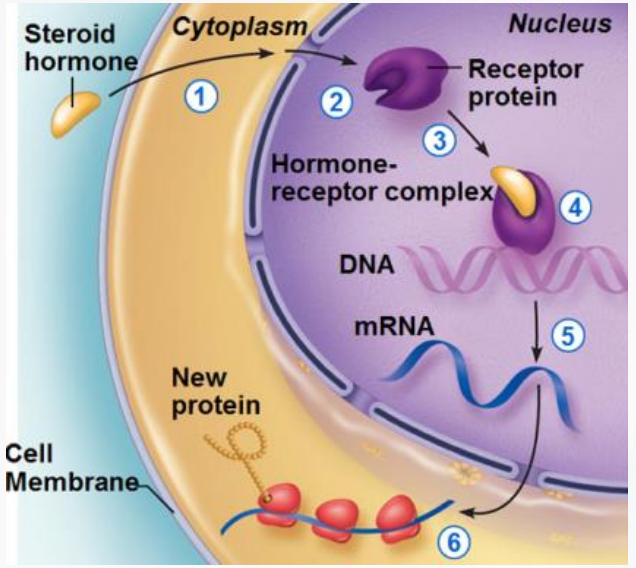
- **On the basis of Mechanism of Action**

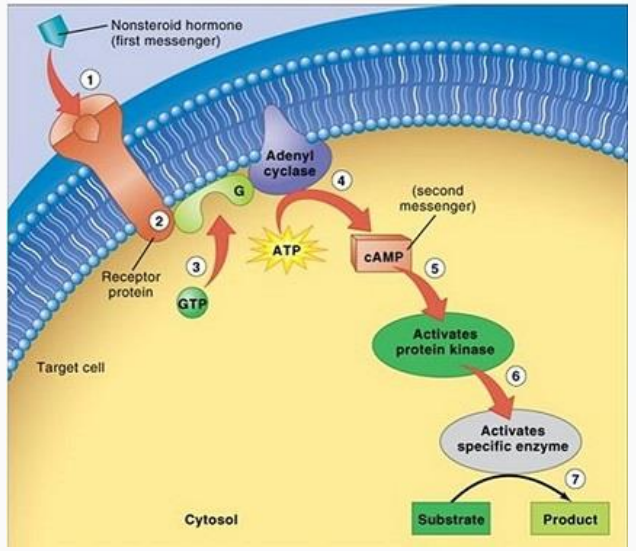
A) <u>Group I Hormones</u>

i) Lipophilic
ii) Bind to intracellular receptors
iii) Bound to transport protein in circulation
iv) Example: Steriod hormones, thyroxine

B) <u>Group II Hormones</u>

i) Hydrophilic
ii) Bind to cell surface receptors
iii) Usually transported in free form
iv) Example: FSH, LH, ACTH, PRL, Insulin





Measurement Of Hormones

- In practice of medicine today, measurement of serum/plasma levels of hormones is practically indispensable.
- It allows evaluation of pathological stage and differential diagnosis of a patient.
- It is also used to monitor and predict a therapeutic success and outcome.
- Bioassays were the first available methods of determining hormone concentrations.
- Discovery of the radioimmunoassay (RIA) for measuring insulin in 1959 opened a new chapter in the history of hormone measurements.
- Competitive and Noncompetitive (Sandwhich) immunoassay being the soul of hormone measurement
- Practical difficulties of handling radioactive material lead to development of various labelling and detection techniques and thus various formats of immunoassays like; ELISA, CLIA, ECLIA

Indications Of Hormone Testing

Reproduction

Test	Indication	
	Male	**Female**
Testosterone-Total	1.Investigation of male hypogonadism. 2.Monitoring of males receiving androgen replacement. 3.Confirming adequacy of anti-androgen therapy in males with prostate carcinoma	1.Investigation of female androgen excess. *Mass spectrometry is sufficiently sensitive to accurately measure the low total testosterone seen in normal females or males rendered chemically castrate for prostate carcinoma as compared to routine immunoassay fromats.
Estradiol (E2) * E2 immunoassay performance is poor at low concentration ranges making these assays inadequate for use in females receiving aromatase inhibitors and in most male patients. E2 analysis by mass spectrometry can be promising for females receiving aromatase inhibitors.	1.Not indicated in routine clinical practice. 2.Investigation for estrogen producing neoplasms in males demonstrating spontaneous feminization including gynecomastia.	1.Investigation of primary ovarian insufficiency or infertility. 2.Monitoring of patients receiving aromatase inhibitor therapy. 3.For monitoring of females undergoing fertility treatment.

Reproduction

Test	Indication	
	Male	**Female**
Leutinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)	1.Investigation of primary or secondary hypogonadism. 2.Investigation of oligospermia or azoospermia. 3.Investigation of infertility. *Usually performed in combination with measurement of appropriate sex steroids (Testosterone for males, E2 for females)	
Prolactin	1.Investigation of infertility / hypogonadism /amenorrhea. 2.Investigation of galactorrhea. 3.Investigation for prolactinoma and other pituitary tumours. 4.Monitoring of prolactinoma in patients treated medically or surgically.	
Dehydroepiandrosterone –sulphate (DHEAS)	Investigation of adrenal mass.	

Adrenal

Test	Indication	
	Male	Female
Cortisol (serum/plasma)	Used in the targeted screening and diagnosis of both Addison's Disease (primary adrenal insufficiency) and Cushing Syndrome. *A morning cortisol measurement can be used to exclude adrenal insufficiency. *A morning serum/plasma cortisol after 1 mg overnight dexamethasone suppression can be used to screen for Cushing Syndrome. *Measurement of cortisol in patients being treated with exogenous glucocorticoids presents numerous bioanalytical and interpretive challenges.	
Adrenocorticotropic Hormone (ACTH)	1.Used in the diagnosis Addison's Disease (primary adrenal insufficiency) and secondary adrenal insufficiency (Tumors in invoving pituitary gland, surgery/ radiation for these tumors). 2.Used to distinguish between Cushing Syndrome caused by adrenal adenoma and Cushing Syndrome caused by inappropriate ACTH production from pituitary adenoma or ectopic source. *For the investigation of adrenal insufficiency, concomitant morning ACTH and cortisol measurement is preferrable.	

Thyroid

Test	Indication	
	Male	**Female**
Thyroid Stimulating Hormone (TSH)	1.Screening for all causes of primary hypothyroidism and hyperthyrodism. 2.Monitoring of patients treated with thyroid hormones / supplementation	
Free Thyroxine (fT4) & Free Triiodothyronine (fT3)	1.Diagnostic confirmation of hyper / hypothyroidism when TSH is abnormal. 2.Assess the severity of hyperthyroidism and ongoing management of Graves' Disease and other forms of hyperthyroidism. 3.Monitor T4 supplementation in patients with secondary hypothyroidism (pituitary cause).	
Total thyroxine/ triiodothyronine (Total T4/T3)	1.Screening for all causes of primary hypothyroidism and hyperthyrodism alongwith TSH. 2.Estimation of free hormones carry a better diagnostic/prognostic value.	

* Calcitonin : Serves as a tumor marker for medullary thyroid cancer and screening those at risk for multiple endocrine neoplasia type 2.

* Thyroglobulin : Serves as a tumor marker for papillary or follicular thyroid carcinoma.

Calcium and Bone Metabolism

Test	Indication	
	Male	**Female**
Parathyroid Hormone (PTH)	1.Diagnostic workup of hypocalcemia and hypercalcemia 2.Analysis of secondary hyperparathyroidism (SHPT), in which PTH concentrations are elevated because of hypocalcemia, hyperphosphatemia, or vitamin D deficiency. SHPT may develop in patients with chronic kidney disease (CKD) and in patients who have undergone bariatric surgery. 3.During and after parathyroid and thyroid surgery.	

* The clinical interpretation of PTH measurements is challenging, because it is complicated by comeasurement of PTH fragments and posttranslationally modified PTH variants, depending on the assay used.

*Both second- and third-generation PTH assays are used in clinical care. In most patients, second-generation assays will measure higher PTH concentrations compared with third-generation assays, because second-generation assays measure C-terminal fragments and third-generation assays do not.

*The observed difference between second- and third-generation assays is most pronounced in patients with CKD because of the impaired renal clearance of PTH fragments.

*During parathyroid surgery, third-generation PTH assays have the advantage of reflecting treatment success more rapidly than second-generation assays.

Glucose Homeostasis	
Test	**Indication**
Insulin	1. Investigation of hypoglycemia. 2. To help distinguish type I diabetes from type II diabetes. 3. Fasting insulin may be useful in the investigation of PCOS in females.
C-peptide	1. Investigation of hypoglycemia.

Growth	
Test	**Indication**
Growth Hormone (GH)	1. To confirm diagnosis and to assess response to treatment in acromegaly by use of dynamic function tests. 2. To assess pituitary ability to generate GH in dynamic function tests for hypopituitarism or pituitary growth hormone deficiency.
Insulin-like Growth Factor 1 (IGF1)	1. To screen for acromegaly and growth hormone deficiency in symptomatic patients. 2. To monitor response to treatment (surgical or medical) for acromegaly and response to therapy in patients receiving recombinant GH.

Challenges Of Hormone Testing

- Immunoassays form an important part of clinical biochemistry laboratory and remain the most commonly used method to evaluate hormonal disorders.

- Due to relatively low concentrations of analyte being measured and because of the complexities of the antigen-antibody interaction, this technique is relatively susceptible to interferences and can distort the clinical picture.

- An interference is defined as the effect of a substance present in the sample that alters the correct value of the result.

- Interferences in immunoassay fall in to 2 broad categories :

1. Analyte - independent

2. Analyte - dependent

Analyte-independent interferences

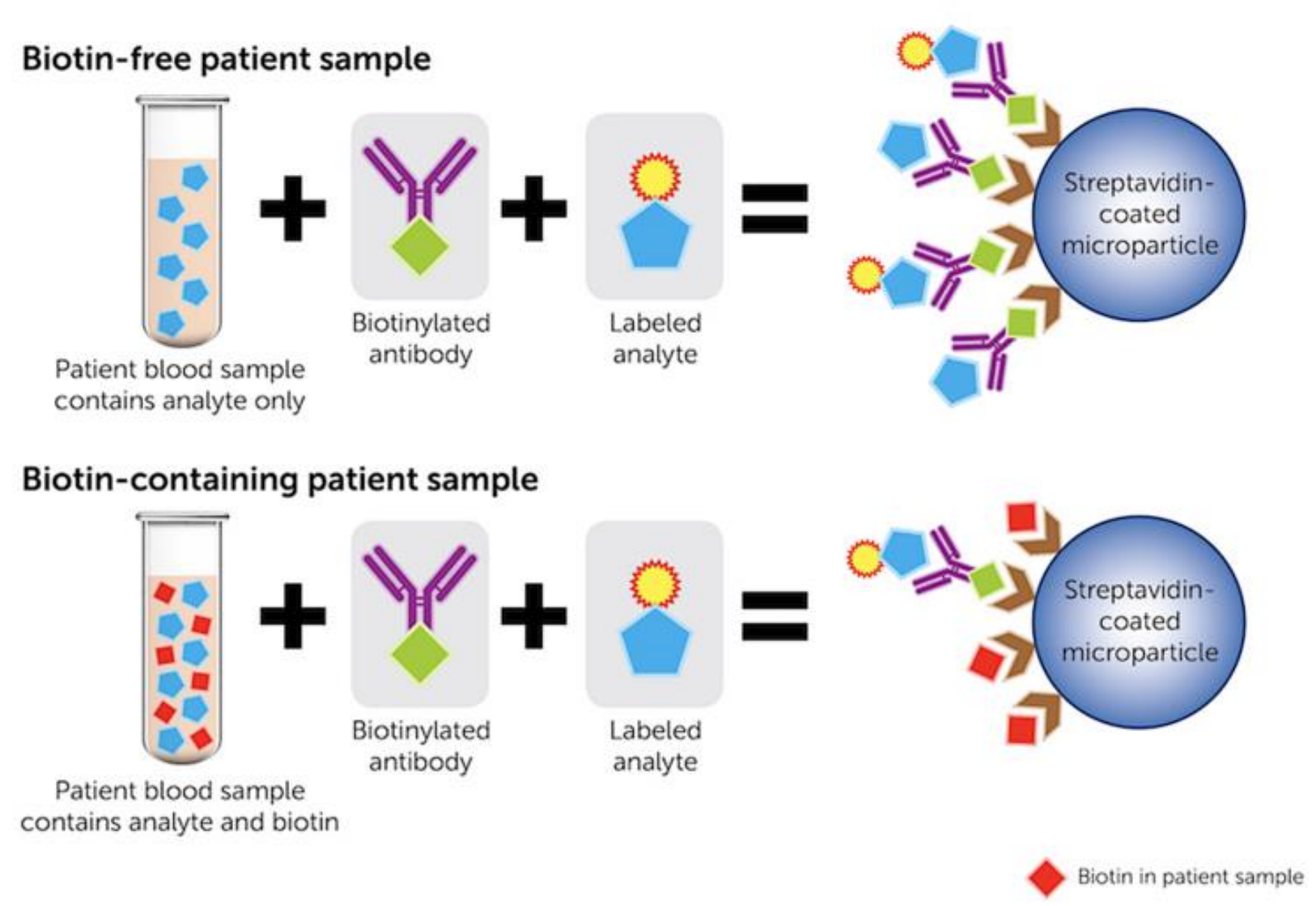
- **Pre-analytical**

○ Inadequate centrifugation with microclots (notorious for generating spurious results)
○ Hemolysis, lipemia, icterus (lipemia can interfere in some immunoassays especially those by nephelometry and turbidimetry)
○ Carryover from very high concentration analyte e.g. hCG or tumour markers
*For most immunoassay analytes, a variety of matrices/sample type are acceptable with a few exceptions such as, ACTH and PTH in EDTA plasma.

- **Analytical**

○ Inadequate separation from binding proteins (thyroxine and cortisol are highly protein bound)
○ Antibodies directed against labels e.g. ruthenium
○ Disease states may cause artifactual changes in analytes e.g. FFA displacing T4 from binding proteins in diabetic ketoacidosis.
○ Interference with signal generation by therapeutic ingestion of similar agent e.g. biotin
*High sr. concentration of biotin can interfere in immunoassay formats utilizing Biotin-Streptavidin technology. Most commonly TFT's are affected.

Interference Of Biotin In Immunoassay Format



Analyte-dependent interferences

- **<u>Cross reactivity</u> (Assay lacks specificity)**

1. Endogenous molecules with a similar structure to the measured analyte exist

- Early hCG immunoassays were cross-reactive with LH. Latest immunoassay formats have resolved this issue.

2. Metabolites of the analyte have common cross-reactive epitopes

- Significant cross-reactivity is noted with assay of immunosuppressant compound cyclosporine A. Large number of metabolites of cyclosporine A depict varying cross-reactivity (levels up to 174% higher in individual patients compared with the HPLC reference method) to the antibodies used in its assay.

3. Administration of structurally similar medications

- Cortisol assays can show significant cross-reactivity with prednisolone and result in false elevated cortisol values in patients using these drugs.

4. Metabolic / Disease states

- Adrenal steroid intermediate like 11-deoxycortisol which is elevated in congenital adrenal hyperplasia and metyrapone therapy (inhibition of steriodogenesis) can interfere with serum cortisol estimation leading false elevated levels of cortisol.

- Endogenous antibodies

1. Reagent / Heterophile antibodies
2. Analyte autoantibody (macro complexes)

1. Reagent / Heterophile antibodies

- Heterophile antibodies (HAB) are antibodies that are formed due to exposure to external antigens. Animal antigens can be involved in forming what is called human anti-animal antibodies. A common antibody that falls in this category is human anti-mouse antibodies (HAMA).
- HAMA can form a bridge between the capture and signal antibodies in “sandwich” immunoassay leading to a falsely elevated signal of the analyte in question.
- Koshida et al. reported an overall prevalence of HAMAs of 11.7% in randomly collected samples.
- Interference noted in TSH, HCG, AFP, CA-125 assays.

Measures to tackle this interference:

- Retesting with a different immunoassay or methodology.
- Addition of heterophile blocking reagent (Non-immune serum, species-specific polyclonal IgG, anti-human IgG or polymerised mouse IgG, non-immune mouse monoclonals, or species-specific fragments of IgG [Fc, Fab, F(ab’)2] from the same species used to produce the reagent antibodies, are commonly used as blocking agents by the manufacturers of kit assays).
- Diluting the sample.

HAMA Interference with Immunoassay

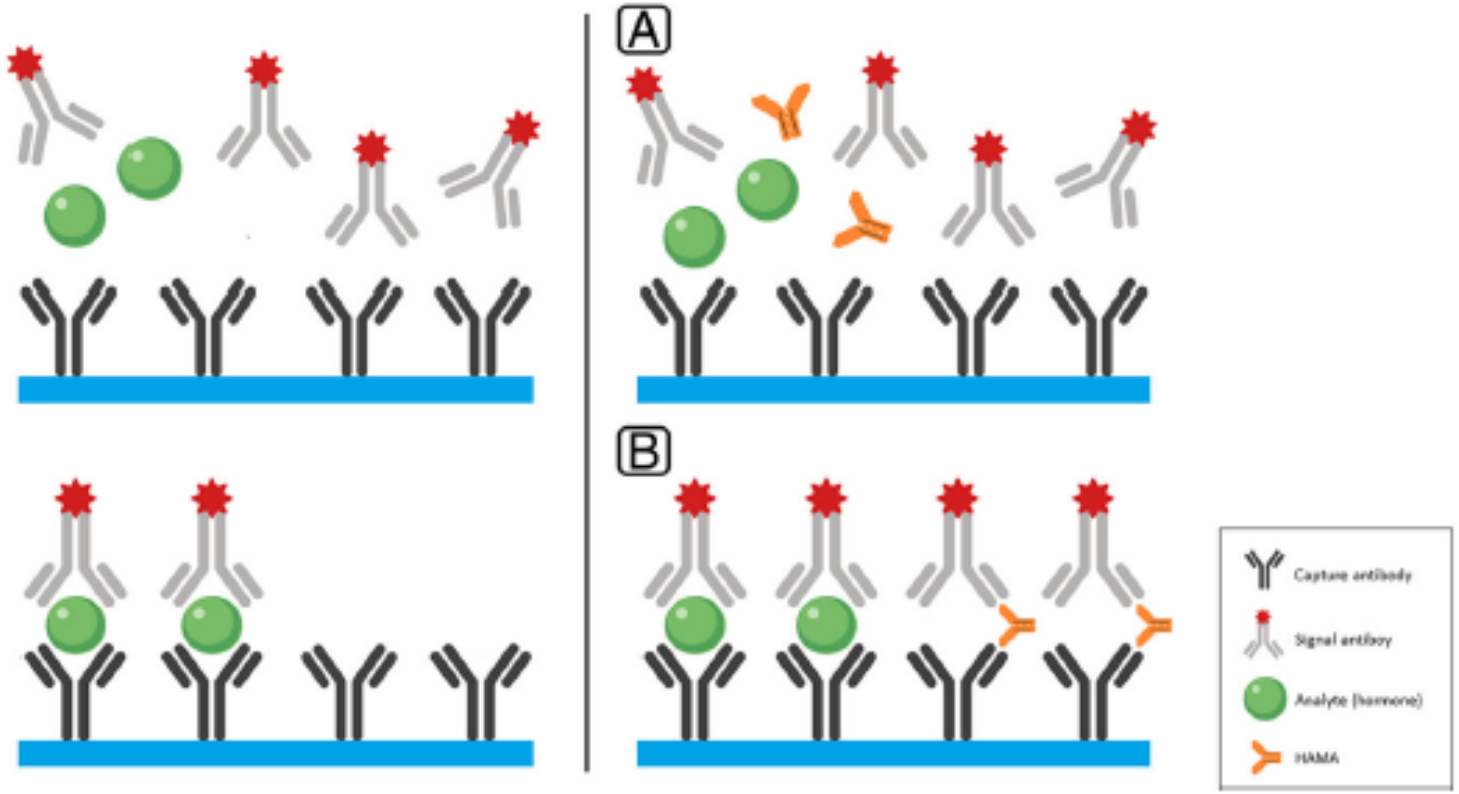


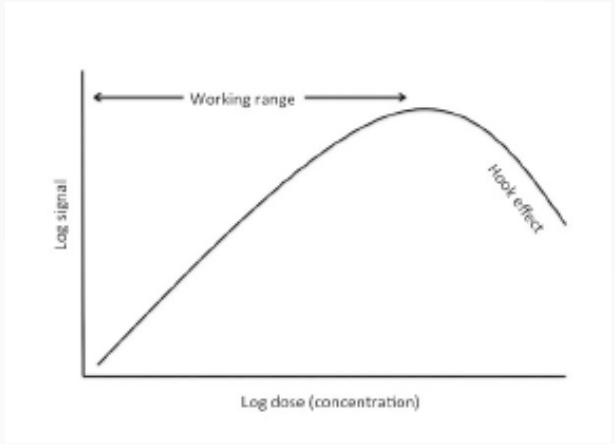
Fig. 4 Illustration of human anti-mouse antibodies (HAMA) interference with immunoassay. The left panel illustrates the non-competitive "sandwich" immunoassay without the presence of HAMA in the sample (see Fig. 2). The right panel illustrates the mechanism of the HAMA interference with immunoassay. **a** The sample containing the studied hormone and the HAMA is added to the test tube which contains both capture and signal antibodies. **b** In addition to the correct formation of "sandwiches" (capture antibody- hormone-signal antibody), the HAMA forms a bridge between the capture antibody and the signal antibody forming antibody-HAMA-antibody "sandwiches". As a result, more signal will be measured, and thus, false elevation of the studied hormone will be reported

2. Analyte autoantibody (macro complexes)

- Autoantibodies are endogenous antibodies directed against the body's own components. Well known examples are macro-prolactin (biologically inactive) which is a complex of IgG and PRL, and anti-thyroid peroxidise (anti-TPO) commonly found in autoimmune thyroiditis.
- Macroprolactin interferes with most immunoassays used for prolactin level measurement leading to falsely elevated prolactin level.
- Clinical scenario lacking clinical and radiological evidence of hyperprolactinemia should trigger macroprolactinemia as the culprit.
- Few studies noted the prevalence of macroprolactinemia as high as 26% of patients with apparent hyperprolactinemia.
- Polyethylene glycol (PEG) precipitation has been indentified as means of rapidly and reliably separating monomeric- and macro-PRL in the laboratory.

- **Hook effect**

- The high dose hook effect can occur when an analyte being measured is present in very high concentrations or the amount of antibodies put by the manufacturer in the kit is low.
- It can occur with 2-site immunometric assays and with nephelometric assays.
- In 2-site assays the very high concentration of analyte may prevent “sandwich” formation with capture and signal antibodies both being bound separately to the analyte, and resultant apparent low concentration of analyte being recorded.
- The shape of the binding curve gave the name “hook effect” to the phenomenon; with gradually-increasing analyte concentrations in the sample, the binding curve goes up, but at some critical point exceeding the capacity of the assay components, it starts “hooking down”.



- The published list of tests susceptible to this hook effect includes;

1. Prolactin in prolactinomas
2. Beta human chorionic gonadotropin (B-HCG) in patients with choriocarcinoma
3. Thyroglobulin in thyroid cancer
4. Prostate-specific antigen in patient with metastatic prostate cancer

- The best possible way of establishing true hormone concentration is to perform a sample dilution 1:100 or even more

Hook Effect

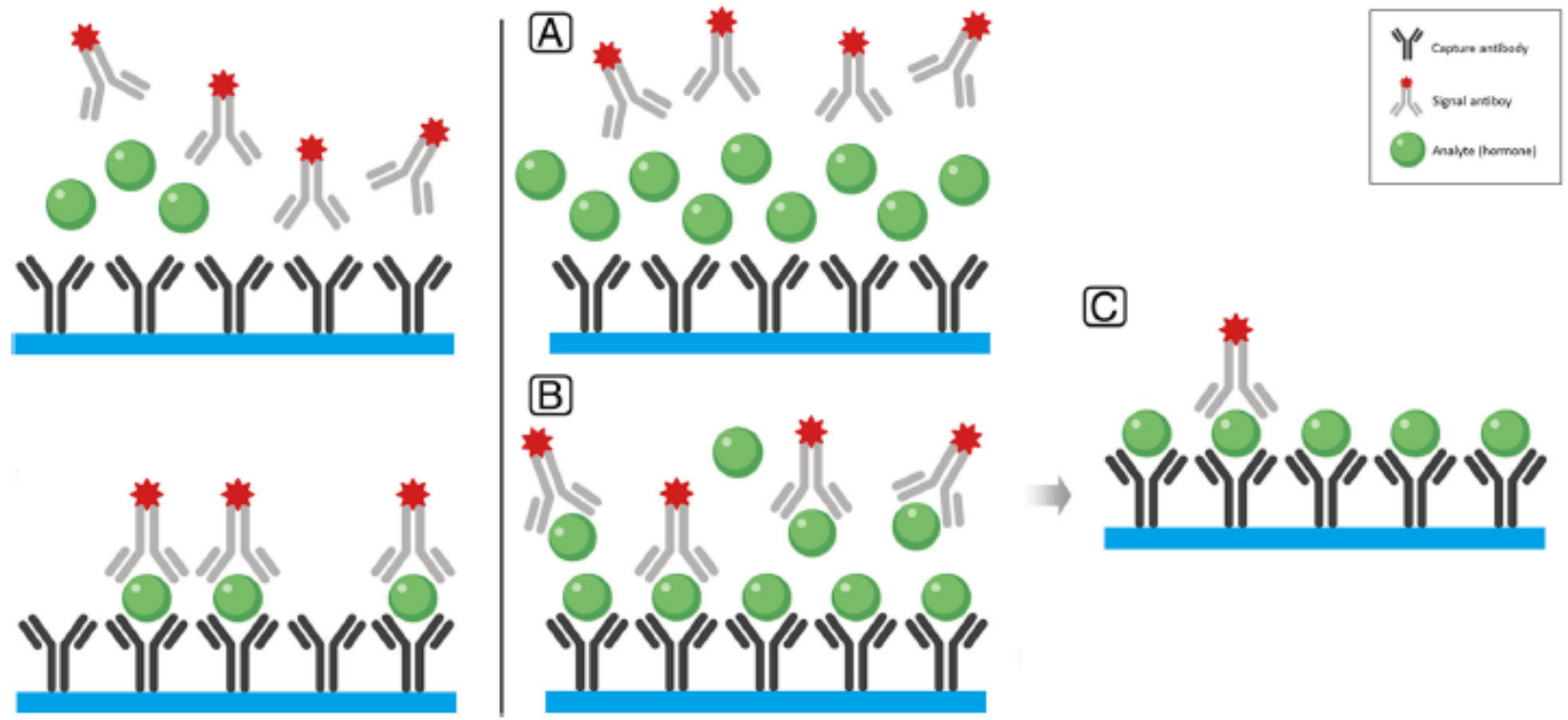


Fig. 3 Illustration of the high dose hook effect. The left panel illustrates the non-competitive "sandwich" immunoassay with normal (or elevated within the tolerance of the assay kit) hormone concentration (see Fig. 2). The right panel illustrates the mechanism of the hook effect with exceedingly high hormone concentration. **a** The sample that contains remarkably elevated hormone concentration is added to the test tube which contains both capture and signal antibodies. **b** The studied hormone overwhelmingly saturates both the capture and signal antibodies preventing the formation of the "sandwiches". **c** After the washout phase, only a few "sandwiches" will be left producing a low signal

Investigation of possible interferences

- A high index of suspicion
- Exclude pre-analytical problems
- Repeat analysis on another instrument from a different manufacturer
- Use of heterophile blocking tubes
- PEG precipitation
- Measurement of dilutions of the sample using the manufacturer's diluent containing non-immune globulin
- Check using a different matrix e.g. urine for hCG
- Column chromatography
- Mass spectrometry

Conclusion

- Despite advances in our knowledge and understanding of the mechanisms of interference in immunoassays, there is no single procedure that can rule out all interferences.
- Laboratory diagnosis should always be done in conjunction with clinical assessment of the patient, including complaints, history of associated diseases, and concomitant medications as well as careful physical examination.
- Any discrepancy between clinical and laboratory data deserves careful attention.
- Laboratory has a particular responsibility to ensure the validity of the results they release.
- For the manufacturers of immunoassay test kits, there must be agreed guidelines for characterizing the effects of interfering substances, continued surveillance for new interfering factors and refinement of immunoassays to render them interference free.

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Thank You