

Chapter 3

Testosterone Assays: Current Techniques and Pitfalls



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Testosterone plays a pivotal role in regulating various physiological processes in males, encompassing metabolic, sexual, cardiovascular, and bone health. Accurate measurement of serum testosterone levels is indispensable for clinical assessment, management, and monitoring by healthcare providers.

Accurate measurement of serum testosterone levels is crucial across different clinical contexts, such as testosterone replacement therapy (TRT) for testosterone deficiency of puberty or late onset hypogonadism and transgender rehabilitation management of endocrine abnormalities. The contemporary clinical approach to diagnosing male hypogonadism relies on accurate serum testosterone levels. Consensus guidelines for testosterone replacement therapy hinge on two main criteria: firstly, the presence of low serum testosterone levels, and secondly, the manifestation of symptoms and signs such as fatigue, cognitive dysfunction, reduced body hair, and mood alterations (Fig. 3.1). Regular laboratory monitoring is essential for patients undergoing testosterone therapy to ensure safety, efficacy, and proper titration to target levels.

Multiple laboratory assays and protocols are available for measuring testosterone, with variations in technologies, protocols, and target ranges among clinical or commercial laboratories. However, standardization of recommended diagnostic and therapeutic ranges remains lacking. Obtaining precise testosterone assays faces challenges due to variations throughout the day, a broad spectrum of normal values, and technical and logistical intricacies during assay execution.

Ensuring the accuracy of serum testosterone level is vital for diagnosing hypogonadism across diverse clinical settings. However, numerous challenges arise, such as inconsistencies in laboratory standardization, reference range variations, and the expenses linked with top-tier equipment. Additionally, technical and logistical

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Which of the following symptoms apply to you at this time? Please, mark the appropriate box each symptom. For symptoms that do not apply, please mark "none".

Symptoms:	None	Mild	Moderate	Severe	Extremely severe
Score =	1	2	3	4	5
1. Decline in your feeling of general well-being (general state of health, subjective feeling)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Joint pain and muscular ache (lower back pain, joint pain, pain in a limb, general back ache)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Excessive sweating (unexpected/sudden episodes of sweating, hot flashes independent of strain)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Sleep problems (difficulty in falling asleep, difficulty in sleeping through, waking up early and feeling tired, poor sleep, sleeplessness)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Increased need for sleep, often feeling tired	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
6. Irritability (feeling aggressive, easily upset about little things, moody)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. Nervousness (nervous tension, restlessness, feeling fidgety)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. Anxiety (feeling panicky)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. Physical exhaustion/lacking vitality (general decrease in performance, reduced activity, lacking interest in leisure activities, feeling of getting less done, of achieving less, of having to force oneself to undertake activities)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. Decrease in muscular strength (feeling of weakness)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. Depressive mood (feeling down, sad, on the verge of tears, lack of drive, mood swings, feeling nothing is of any use)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. Feeling that you have passed your peak	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
13. Feeling burnt out, having hit rock-bottom	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
14. Decrease in beard growth	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
15. Decrease in ability/frequency to perform sexually	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
16. Decrease in the number of morning erections	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
17. Decrease in sexual desire/libido (lacking pleasure in sex, lacking desire for sexual intercourse)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Have you got any other major symptoms?	Yes <input type="checkbox"/>	No <input type="checkbox"/>			
If Yes, please describe:					

Ageing Male Symptom (AMS) score

Assesses sexual, psychological & somatic complaints

Severity does not necessarily correlate with testosterone level.

No single symptom pathognomonic of hypogonadism

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Fig. 3.1 Aging male symptom score questionnaire. (Reproduced with permissions from Heinemann et al. [1]. Copyright © 2003 Heinemann et al.; licensee BioMed Central Ltd. Open Access)

obstacles further complicate assay procedures. This chapter aims to explore the diverse array of testosterone assays and their interpretation to provide clarity in clinical practice.

The Testosterone Equation

Total testosterone levels encompass testosterone bound to several proteins, including sex hormone-binding globulin (SHBG), albumin, corticosteroid-binding globulin, and orosomucoid, as well as a fraction of unbound hormone. The free hormone hypothesis proposes that the unbound fraction is the biologically active component, although this concept is debated. Some studies indicate that the bioavailable fraction, consisting of free testosterone and nonspecifically bound testosterone, more accurately reflects biological activity compared to free testosterone alone.

Total Testosterone = [SHBG bound testosterone] + [Albumin bound testosterone] + [Free Testosterone].

Bioavailable Testosterone = [Albumin bound testosterone] + [Free Testosterone].

Testosterone Measurements

Testosterone measurement poses significant challenges in standard clinical settings, with various methods available for assessing total testosterone levels, including immunoassays and mass spectrometry. Immunoassays, although cost-effective and technically straightforward, are surpassed by mass spectrometry, considered the gold standard despite its higher cost and technical complexity. Free testosterone measurement methods include equilibrium dialysis, immunoassays, or calculations using algorithms based on total testosterone, SHBG, and albumin levels. However, equilibrium dialysis, while the gold standard, is limited by technical demands and time constraints, making it impractical in most laboratories. Research highlighted significant disparities in assay accuracy and precision among laboratories, mainly due to calibration inaccuracies and insufficient analytical specificity. Programs like the CDC Hormone Standardization Program seek to accredit laboratories and assays, promoting uniformity and dependability in testosterone measurements. Addressing these challenges, the American Urological Association (AUA) recommends consistent total testosterone assessment at one laboratory using the same assay repeatedly, ideally with morning samples, to ensure result consistency and comparability.

Total Testosterone Measurements

Immunoassays

Immunoassays are commonly used to measure total testosterone levels, utilizing tracer-linked testosterone molecules that compete with native testosterone for binding to testosterone antibodies. These tracer molecules can be enzymes, radioisotopes, and chemiluminescent or fluorescent compounds. (Fig. 3.2). Despite their prevalence, immunoassays present drawbacks, necessitating technical proficiency, involving time-intensive extraction/chromatography procedures, and exhibiting diminished accuracy at extreme testosterone concentrations. Moreover, radioimmunoassay entail the generation of radioactive waste, further complicating their utilization.

Mass Spectrometry

The Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) technique involves several key steps (Fig. 3.3) in analyzing testosterone-containing samples. Initially, the samples undergo preprocessing and enrichment to enhance the concentration of testosterone. Following this, the testosterone compounds are separated using liquid chromatography (LC). Subsequently, ionization occurs either directly

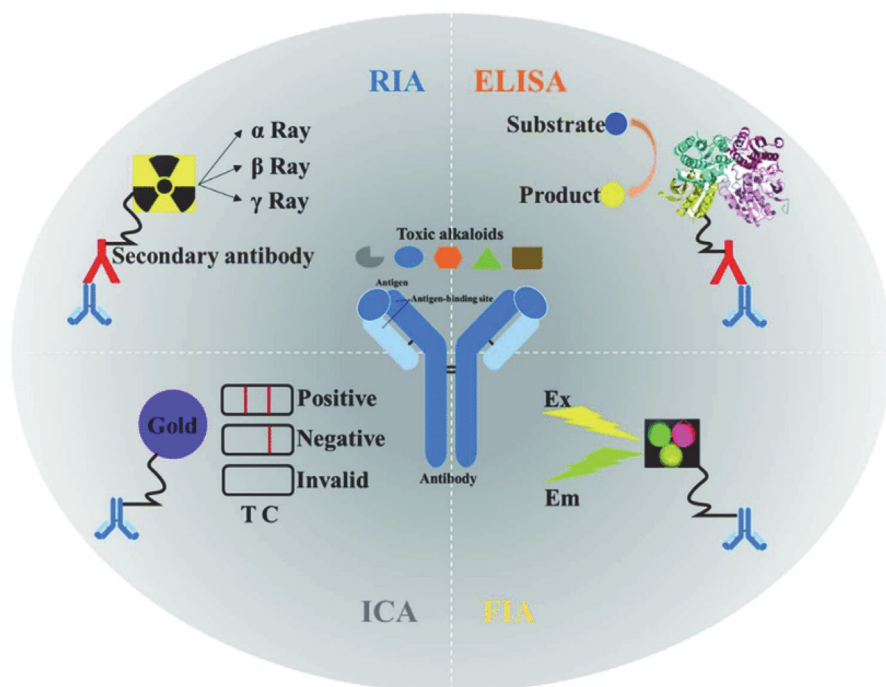


Fig. 3.2 Application and schematic diagram of four types of immunoassays for testosterone immunoassay. *RIA* radioimmunoassay, *ELISA* enzyme-linked immunosorbent assay, *ICA* immuno-chromatography assay, *FIA* fluoroimmunoassay, *Ex* excitation, *Em* emission, *T* test line, *C* control line. (With permissions from Ren et al. [3]. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>))

through electrospray ionization (ESI) or via spotting with matrix-assisted laser desorption ionization (MALDI). In the first mass analyzer (ion trap), precursor ions are selected and then fragmented using methods such as collision-induced dissociation (CID) or higher energy collisional dissociation (HCD). The resulting ions proceed to the second mass analyzer (e.g., orbitrap), where they undergo high-resolution measurement using bioinformatics tools. This comprehensive process ensures accurate and detailed analysis of testosterone levels in the sample.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) has risen as the favored method for total testosterone level measurement, establishing itself as the gold standard in clinical settings. Despite its higher cost, LC-MS/MS offers superior sensitivity and specificity compared to immunoassays, especially in detecting both low and high testosterone levels where IAs may show considerable variability. The adoption of LC-MS/MS assays has experienced substantial growth recently, highlighting the increasing recognition of its advantages in clinical practice. Despite some inter-laboratory variability; MS signifies a significant

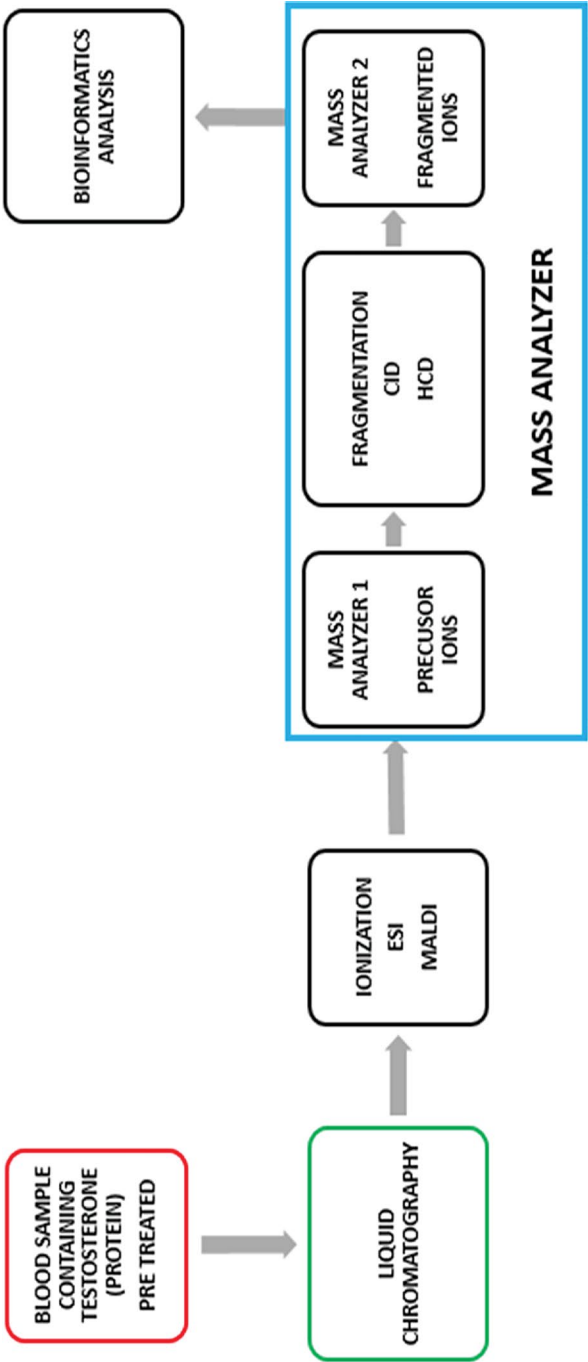


Fig. 3.3 Principle of Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

advancement over commercially available immunoassays in terms of reliability and accuracy.

Immunoassay Vs. Mass Spectrometry for Measuring Total Testosterone

Research conducted by Wang et al. and Taieb et al. assessed the precision of various automated and manual Immunoassay methods, contrasting them with the gold standard liquid chromatography-tandem mass spectrometry. Neither study concluded that Immunoassay techniques were adequately precise at detecting low serum testosterone levels. Despite these limitations, specific Immunoassay methods might remain appropriate for diagnosing hypogonadism and guiding treatment choices in adult males with decreased testosterone levels. Some studies indicate that Immunoassay methods lack consistency in measuring low testosterone levels in women and children compared to mass spectrometry analysis, underscoring challenges in their suitability across diverse patient demographics.

Free Testosterone Measurements

Equilibrium Dialysis

Equilibrium dialysis stands out as the gold standard technique for free testosterone detection. This method employs a semi-permeable dialysis membrane (Fig. 3.4) to separate two compartments: one containing plasma with protein-bound and free testosterone, and the other containing phosphate buffered saline (PBS). The dialysis membrane serves as a molecular sieve, permitting free testosterone to pass through while retaining plasma proteins and testosterone-protein complexes in the donor compartment. The calculation of free testosterone can then be conducted through either direct methods, such as LC-MS/MS or immunoassays, or indirect methods involving a small quantity of testosterone radiotracer. The accuracy of this measurement significantly hinges on the analytical performance during the measurement step.

Challenges associated with equilibrium dialysis include extensive time for sample dilution and processing and susceptibility to factors like temperature and pH, emphasizing the need for a standardized environment. A modified approach called ultrafiltration, utilizing centrifugation to expedite the sample's movement through the membrane, has been developed. While this method reduces operating times significantly and offers comparable analytical performance to standard equilibrium dialysis, it may encounter issues such as sample adsorption to the ultrafiltration filter, which varies depending on the commercial filter used.

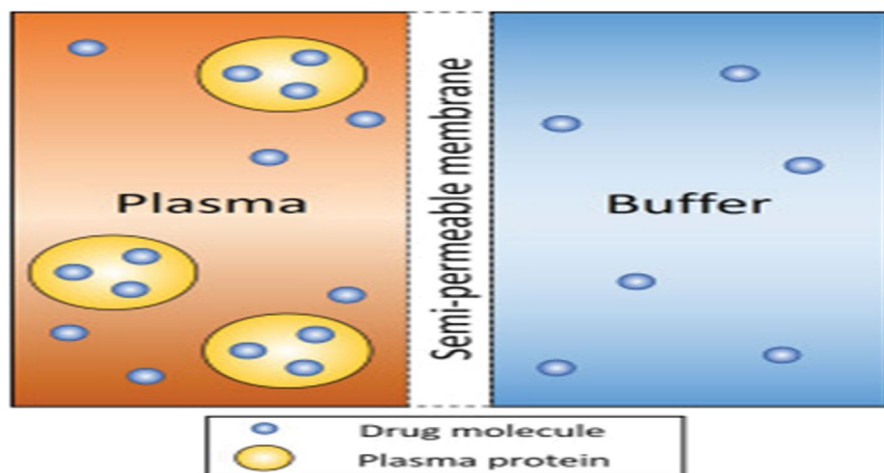


Fig. 3.4 Principle of equilibrium dialysis. (Permission from: Hann et al. [4])

Analog Immunoassays

Analog Immunoassays (IAs) offer similar advantages as discussed previously, being readily available in both large and small laboratories, cost-effective, and quick to perform. However, despite their accessibility, IAs may yield inaccurate measurements of free testosterone due to fluctuations in SHBG levels, especially considering the relatively low concentrations of free testosterone. Consequently, utilizing IAs for free testosterone measurement is generally discouraged.

Calculated Free Testosterone

Indirect methods for assessing free testosterone involve utilizing established algorithms to estimate bioavailable testosterone, free androgen index (FAI), and free testosterone index (FTI). These algorithms, including the Sodergard, Nanjee-Wheeler, Vermeulen, and Ly-Handelsman approaches, utilize measured concentrations of testosterone, albumin, and sex hormone-binding globulin (SHBG) to calculate free testosterone levels. Despite some scrutiny regarding their predictive accuracy, studies such as that conducted by Morris et al. have demonstrated their efficacy in assessing biochemical hypogonadism. Through regression analysis, Morris et al. derived an equation for bioavailable testosterone that showed a strong correlation with true values.

The precision of the calculated free testosterone value depends on the accuracy of total testosterone, SHBG, and albumin measurements. Several algorithms have been developed based on equilibrium binding principles or empirical modeling using known concentrations of analytes.

Bioavailable Testosterone Measurements

Ammonium Sulfate Precipitation Method

This is utilized to assess bioavailable testosterone levels by mixing tracer-labeled testosterone with serum. This mixture is then treated with a saturated solution of ammonium sulfate, causing precipitation of sex hormone-binding globulin (SHBG). The remaining tracer-labeled testosterone can then be quantified, and when multiplied by total testosterone levels, provides an estimation of bioavailable testosterone in serum. While this method generally correlates well with equilibrium dialysis, it is subject to limitations such as reliance on the accuracy of the total testosterone assay and potential interference from tracer impurities.

Concanavalin A Separation Method

An alternative method to SHBG precipitation using ammonium sulfate is concanavalin A separation. This approach is suggested as a more specific alternative, aiming to address issues related to nonspecific albumin precipitation in poorly controlled assay conditions. A study conducted by Giton et al. compared bioavailable testosterone measurements using both techniques and found comparable results. However, further assessment is necessary before considering the widespread adoption of concanavalin A separation in routine clinical practice.

Testosterone Reference Ranges

The absence of a universally recognized standard reference range for testosterone levels in healthy men highlights the variability and lack of consensus regarding lower testosterone thresholds. Precise reference ranges are essential for the accurate diagnosis of conditions such as hypogonadism. Table 3.1 outlines the recommended cutoff ranges suggested by medical societies.

Age-Specific Reference Ranges

The association between aging in healthy men and declining serum testosterone concentrations has been a topic of debate for some time. Numerous studies indicate a decrease in serum testosterone levels from the fourth to fifth decade of life, declining by approximately 1–2% per year. Research suggests that the mean serum

Table 3.1 Different medical societies propose varying lower threshold limits for testosterone levels

Medical society	Testosterone cutoff value
American Society of Andrology (ASA) International Society for the Study of Aging Male (ISSAM) International Society of Andrology (ISA) European Association of Urology (EAU) European Academy of Andrology (EAA)	230 ng/dl (8 nmol/L)
Endocrine Society	280–300 ng/dl (9.7 to 10.4 nmol/L)
American Urological Association (AUA)	<300 ng/dl (<10.4 nmol/L)

testosterone levels at 75 years of age are roughly two-thirds of those observed at 25 years of age.

In a study conducted by Zhu et al. in 2022, researchers analyzed the testosterone levels of 1486 men aged between 20 and 44. Their approach involved defining normal testosterone levels by selecting the middle third of the data range, aligning with the American Urological Association’s definition of normal testosterone levels. However, this method of determining normal ranges might result in overly narrow reference intervals. As more data accumulates, it’s anticipated that testosterone reference ranges will evolve to incorporate age-specific values.

In a cross-sectional cohort study by Zitzmann et al. in 2006 involving 434 men, it was observed that specific symptoms were more prevalent as testosterone levels declined below certain thresholds:

- Reduced energy levels were more likely when testosterone levels fell below 15 nmol/L.
- Weight gain became more prevalent with testosterone levels below 12 nmol/L.
- Low mood was more commonly reported when testosterone levels dropped below 10 nmol/L.
- Erectile dysfunction was observed to increase when testosterone levels fell below 8 nmol/L.

Nevertheless, focusing on the overall trend of testosterone levels and the presence of associated symptoms may hold greater significance. Even if an individual falls within the lower end of the normal range without experiencing symptoms, it could still be considered normal for them.

Utilizing a uniform reference range for all age brackets might lead to an overestimation of low testosterone prevalence among older individuals when contrasted with age-specific criteria. Laboratory standards frequently fail to account for age-related fluctuations in testosterone consistently. This inconsistency is further complicated by the age-linked elevation in sex hormone-binding globulin (SHBG), intensifying the decline in bioavailable testosterone among aging men. Notably, findings from the Massachusetts Male Aging Study revealed a 2–3% annual reduction in bioavailable testosterone. Hence, the establishment of age-specific reference ranges is imperative for precisely evaluating testosterone levels across diverse age cohorts.

Standardization of Reference Ranges

Establishing consistent reference ranges for testosterone levels presents challenges due to variations influenced by biological, environmental, and methodological factors. Discrepancies between assays and laboratories further complicate this process, along with the impact of diseases and medications on testosterone levels.

Efforts to standardize assay technologies aim to address these challenges by minimizing differences between systems. Normalizing equations, calibrated to a reference method, help reduce variation between cohorts, facilitating the calculation of unified reference ranges applicable across different laboratories. However, setting standardized reference ranges requires a comprehensive understanding of the biological and social factors influencing testosterone distribution.

Validation through data from randomized trials and longitudinal studies is essential to ensure the clinical suitability and reliability of standardized reference ranges. This rigorous approach ensures that reference ranges accurately reflect testosterone levels across diverse populations, supporting effective clinical decision-making.

What Time to Suggest for Laboratory Testing?

The testosterone secretion becomes nyctohemeral in adulthood, i.e., cyclic event occurring during both day and night. Testosterone is produced in the range of 6–7 g/day in a damped irregular and pulsatile manner. It usually peaks and nadirs at fall and spring and morning and evening respectively, and it peaks at the second and third decade of life.

The fluctuation of testosterone levels throughout the day exhibits a less pronounced pattern in older men compared to their younger counterparts. Research indicates a significant difference of 20–25% in testosterone levels between morning and afternoon among younger men aged 30–40, whereas this difference diminishes to about 10% in men aged 70.

For precise assessment of peak morning levels, it has been recommended to collect testosterone samples between 07:00 and 09:00. This timing optimizes the assessment of testosterone status, facilitating reliable diagnosis and monitoring of conditions associated with testosterone levels.

Frequency/Follow-Up Laboratory Testing

Ensuring the accuracy of testosterone level assessments involves validating laboratory values with at least two readings. This step is crucial because many men initially diagnosed with low testosterone levels were later found to have normal values upon retesting, emphasizing the need for confirmation.

It is worth noting that repeat measurements can exhibit significant variability, ranging from 65% to 153%, depending on the assay used. Therefore, performing

two or more repeat measurements can help reduce some of this variability and offer a more precise evaluation of testosterone status.

Conclusions

In conclusion, diagnosing hypogonadism and optimization of testosterone therapy requires precise measurement of serum testosterone levels. Despite challenges such as inconsistencies in assay standardization, variations in reference ranges, and technical complexities, notable advancements have been achieved in improving our understanding and approach to testosterone measurement.

Various methods, including immunoassays and mass spectrometry, are available for quantifying total testosterone levels, each with its advantages and limitations. While immunoassays are widely accessible and relatively cost-effective, mass spectrometry is recognized as the gold standard due to its superior accuracy and specificity, particularly at extreme testosterone levels. Furthermore, the measurement of free testosterone, although technically demanding, is essential for a comprehensive evaluation of androgen status.

Efforts to standardize reference ranges and harmonize assay technologies have enhanced the consistency and reliability of testosterone measurements across different laboratories and populations. However, ongoing research and validation are necessary to ensure the clinical relevance and applicability of standardized reference ranges, especially in diverse demographic groups and clinical settings.

Additionally, considerations such as the timing of laboratory testing and the importance of repeat measurements play a vital role in accurately interpreting testosterone levels and making informed clinical decisions.

In summary, despite the complexities and challenges involved in testosterone measurement, continued advancements in technology, standardization efforts, and research endeavors contribute to improving the accuracy and utility of testosterone assays in clinical practice. By addressing these challenges and leveraging emerging technologies, healthcare providers can effectively diagnose and manage testosterone-related conditions, ultimately enhancing patient care and outcomes.

Further Reading

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