Quest Journals
Journal of Medical and Dental Science Research
Volume 12~ Issue 6 (June 2025) pp: 77-81
ISSN(Online): 2394-076X ISSN (Print):2394-0751
www.questjournals.org

Research Paper

The contribution of liquid chromatography coupled to tandem mass spectrometry (LC MS/MS) in the analysis of testosterone

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Abstract:
The combination of liquid chromatography coupled to tandem mass spectrometry (LC MS/MS) has significantly improved testosterone analysis, offering an analytical method with high sensitivity, specificity and also reproducibility suitable for many applications. This approach is particularly relevant in clinical diagnostics, sports science and the control of substances in dietary supplements. The integration of LC-MS/MS enables accurate and reliable quantification of testosterone concentrations, alleviating the limitations inherent in conventional immunoassays.
Testosterone is a key hormone involved in the regulation of numerous physiological processes in both men and women. Accurate analysis of this hormone is crucial for the diagnosis of endocrine dysfunctions, such as polycystic ovary syndrome and hypogonadism, as well as for routine testing. Testosterone concentrations have traditionally been assessed using serum or plasma samples, an approach that presents challenges in terms of collection, storage and transport, particularly in regions with limited resources.
Measurement of testosterone using immunoassays has proved imprecise, particularly at low concentrations, due to interference from cross-reactivity with structurally related steroid hormones or synthetic derivatives. Immunoassays are widely used in clinical laboratories for the measurement of testosterone in serum/plasma, although second-generation assays have improved considerably, liquid chromatography-tandem mass spectrometry, LC-MS/MS, has been recommended as a better alternative, due to its high specificity and accuracy and its ability to measure over a wide range of concentrations.
The use of dried blood drops (DBS) has emerged as an optimized strategy for hormone analysis, offering significant advantages in terms of sample stability, ease of collection and streamlining of logistical processes.
The aim of this study is to optimize the contribution of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in testosterone analysis, to ensure validity and accuracy comparable to conventional serum methods.

Key words: *testosterone, mass spectrometry, liquid chromatography, chemiluminescent immunoassays, dried blood drop (DBS), conventional immunoassays.*

***R**eceived 02 June., 2025; **R**evised 09 June., 2025; **A**ccepted 11 June., 2025 © The author(s) 2025.*
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I. Introduction

Testosterone is a primordial androgenic hormone, playing a crucial role in the development of male reproductive tissues, the maintenance of secondary sexual characteristics, and the regulation of multiple physiological functions in both men and women. In particular, it is essential for reproductive health, muscle mass, bone density and general well-being [1,2]. Synthesized from cholesterol or converted from precursors such as dehydroepiandrosterone (DHEA), testosterone circulates mostly bound to sex hormone-binding globulin (SHBG)

or albumin, while a small free fraction (around 2-3%) constitutes the biologically active form. In men, measurement of total testosterone is essential for diagnosing and monitoring pathologies such as hypogonadism and prostate cancer. In women, testosterone serves as a precursor to estradiol, and high circulating levels are associated with disorders such as hirsutism, virilization or oligomenorrhea. In children, testosterone levels can be used to diagnose early or delayed puberty [3]. Accurate and reliable testosterone levels are therefore essential in the clinical setting, particularly for the diagnosis of various endocrine pathologies such as polycystic ovary syndrome. However, this quantification remains complex due to the presence of structurally related steroid hormones, a wide range of physiological concentrations, and low levels in women and children[3] .

Historically, total testosterone was determined by competitive immunoassay using chemiluminescent microparticles (CMIA). This technique, based on anti-testosterone monoclonal antibodies, remains suitable for adult men, whose levels generally exceed 230 ng/dL . Nevertheless, it suffers from low specificity and limited accuracy for concentrations below 100 ng/dL, which restricts its use in female and paediatric populations[3] .

In addition, traditional assays performed on serum or plasma, although standardized, present technical and logistical constraints linked to sample collection, storage and transport. These difficulties are particularly acute in resource-limited settings or for large-scale epidemiological studies. To overcome these limitations, alternative methods such as dried blood spot (DBS) analysis have emerged, mainly using liquid chromatography-mass spectrometry (LC-MS/MS) techniques [4,5].

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is now establishing itself as the reference method for quantifying testosterone in various biological matrices, notably serum and saliva, thanks to its superior sensitivity and specificity [3,6,7].

This analytical advance enables accurate and reliable quantification, essential for the diagnosis and management of androgenic disorders in men, women and children, as well as for forensic investigations linked to the abuse of anabolic steroids [6].

The aim of this article is to present and analyze the contribution of Liquid Chromatography coupled to Tandem Mass Spectrometry (LC MS/MS) in the determination of testosterone, highlighting its advantages over conventional immunological methods, particularly in terms of sensitivity, specificity and reliability, as well as its potential in different biological matrices and clinical contexts.

Principle of the technique :

Liquid chromatography coupled to tandem mass spectrometry (LC MS/MS) is a powerful analytical technique with very high sensitivity and specificity, which integrates the separation capabilities of liquid chromatography with the analytical capabilities of mass spectrometry [8]. This technique requires prior sample preparation. This begins with the addition of a labeled internal standard, such as testosterone-16,16,17-d_3, to correct analytical variations. Testosterone is then extracted from serum by methods such as liquid-liquid extraction or protein precipitation.

Separation by liquid chromatography upstream of the mass spectrometer is a critical step in the analysis, since it must efficiently separate molecules of the same mass (isobars) and potentially yielding the same fragments. This stage is very often carried out using apolar columns (C8, C18), with increasing use being made of UHPLC (working pressures of up to 1,000 bar), which offers gains in sensitivity, resolution, separation speed and solvent consumption. The mass spectrometers used for steroid assays mainly comprise an ionization source, a triple-quadrupole and a detector. The types of ionization frequently used in steroidal hormonology are electrospray (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI). Ionized molecules are directed to the first quadrupole, which selects ions according to their mass-to-charge ratios (parent ions, m/z). The second quadrupole is a collision chamber in which the selected ions are fragmented into daughter ions. The ions resulting from the fragmentation are again selected according to their mass-to-charge ratios by the third quadrupole. The use of these three quadrupoles is known as tandem spectrometry. The sorted daughter ions are then directed to the detector. The monitoring of several parent/child ion transitions constitutes the MRM (multiple reaction monitoring) mode of analysis. Software packages (such as Analyst® or MassLynx®) can be used to process data obtained inMultiple Reaction Monitoring (MRM) mode. In this way, the molecular filter provided by the mass spectrometer brings selectivity to the assay, as the parent- son ion transition is specific to the molecule being assayed. In the case of isobaric molecules with similar structures and likely to produce the same transitions, chromatographic separation is essential. The combination of chromatographic separation and mass spectrometry means that several biological parameters can be assayed selectively on the same sample, in a single analysis. In addition, the determination of steroids requires the selection of a sufficiently sensitive instrument, the sensitivity of the spectrometer being partly dependent on its acquisition capacity (number of scans acquired during a chromatographic peak), the number of molecules fragmented and therefore the number of ions analyzed [8] .

Applications of LC-MS/MS in testosterone analysis:

The integration of Liquid Chromatography coupled to Tandem Mass Spectrometry (LC-MS/MS) has significantly improved testosterone analysis by providing a robust, precise, highly sensitive, specific and reproducible analytical method suitable for a wide range of applications. This technique is a benchmark in fields such as clinical diagnostics, sports science and the control of substances in dietary supplements. The combination of LC-MS/MS enables precise quantification of testosterone levels, overcoming the limitations associated with traditional immunoassays. Among the main contributions of LC-MS/MS to testosterone analysis are:

1- diagnostic role :

Testosterone plays a central role in the regulation of many physiological processes. Accurate measurement is essential for diagnosing endocrine disorders such as hypogonadism or polycystic ovary syndrome [2].

LC-MS/MS enables the simultaneous quantification of several steroid hormones (estradiol, progesterone, testosterone) in different matrices, even at low endogenous concentrations, which is crucial in monitoring pathologies such as infertility, osteoporosis or certain hormone-dependent cancers. Furthermore, rapid and simple methods have been developed for the simultaneous analysis of testosterone, androstenedione and DHEA in adult serum, with minimal sample preparation [7,9].

Dried blood drop (DBS) analysis coupled with LC-MS/MS has been validated, demonstrating excellent linearity, accuracy and clinical correlation with venous blood assays, making it a reliable tool suitable for resource-limited settings [2].

LC-MS/MS steroidometry also enables in-depth characterization of steroid biosynthetic pathways and their metabolic interactions. Studies have confirmed the sensitivity and versatility of this method for quantifying endogenous steroids in serum, with high accuracy and specificity[10].

A study by Gjorgoska et al. presented a rapid and versatile liquid chromatography-tandem mass spectrometry (LC-MS/MS) method designed to accurately quantify endogenous steroids in human serum. And it showed sufficient sensitivity of the method to reliably quantify all targeted steroids at levels typically found in circulation, with the exception of 11OHP4 and 11KP4, which were within acceptable limits for bioanalytical method validation [10].

A study by Yvonne Lood et al. developed and validated the LC-MS/MS method for the determination of testosterone in serum and saliva simultaneously, detecting a wide range of serum concentrations measured in clinical as well as forensic samples. In concluding that the analytical method with selective chromatography combined with specific tandem MS, was intended for routine clinical applications, controlled studies and forensic toxicological investigations[6].

The use of liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in the analysis of sex steroids such as testosterone has proved effective in patients with castrated prostate cancer (PCa) and postmenopausal women[11]. The study by Rao et al. aimed to develop and validate LC-MS/MS techniques for blood sex steroids and compare them with commonly used automated immunoassays (AIA), and concluded that LC-MS/MS assays offer improved sensitivity and specificity over AIA, and have the potential to reveal new clinical applications in castrated PCa patients and postmenopausal women[11].

Testosterone quantification plays an important role in the differential diagnosis of androgen-related endocrine diseases. Mass spectrometry has higher precision and lower variability than immunoassays, particularly at low testosterone concentrations[12] . A study by Sun,G.,Xue et al. developed and validated a high-performance liquid chromatography tandem mass spectrometry method for the determination of human serum testosterone. The study validated the new method, demonstrating high precision and accuracy. The intra-assay coefficient of variation ranged from 1.40% to 2.77%, while the inter-assay coefficient varied from 3.06% to 3.66%. The method also showed a testosterone recovery rate of between 94.32% and 108.60%, indicating its reliability for clinical use. The method developed is suitable for routine clinical practice, enabling accurate measurement of testosterone levels in both men and women. This advance could significantly improve the diagnosis and treatment of androgen-related disorders [12].

The identification of hyperandrogenism in polycystic ovary syndrome (PCOS) is of concern due to the low accuracy of androgen immunoassays (AI) and controversies over which androgens to measure. A study conducted by a illustrates the impact of testosterone (T) and androstenedione (A) assessment by liquid chromatography in tandem with mass spectrometry (LC/MS-MS), in the diagnosis of PCOS. The results confirm that LC-MS/MS is slightly more sensitive than AI in the diagnosis of PCOS, with LC-MS/MS detecting higher levels of fT and A. In addition, assessment of fT and A by LC-MS/MS had a similar level of accuracy in distinguishing PCOs from control subjects. Finally, fT by LC-MS/MS correlated with unfavorable metabolic parameters[13].

2-Sport science:
A rapid and accurate LC-MS/MS method has been developed for the simultaneous quantification of total and free testosterone in serum, validated in elite athletes and demonstrating concentrations above those of the general population. These biomarkers are essential for monitoring anabolic metabolism and early detection of overtraining syndrome (OTS). LC-MS/MS offers a reliable alternative to immunoassays, with improved sensitivity and specificity, facilitating performance monitoring and training management [14].

3- Detection in health supplements:
LC-MS/MS is also used to detect the illegal addition of testosterone to health supplements, a common practice to artificially enhance the efficacy of these products. This method guarantees consumer safety and regulatory compliance by accurately identifying the presence of prohibited hormones [15].
Moreover, testosterone is frequently used as a doping substance, and LC-MS/MS enables reliable analysis in clinical and forensic contexts, with limits of quantification adapted to international standards [6].

4- Sample multiplexing :
For high-volume assays, optimizing throughput reduces assay costs and turnaround times. One approach to liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques is sample multiplexing, in which the analyte of interest is derivatized in different samples with reagents of different molecular weight (differential mass labeling). Samples can then be combined and analyzed simultaneously within a single injection to improve throughput [16].

5-Sensitivity and specificity:
LC-MS/MS methods achieve lower limits of quantification as low as 0.058 ng/mL, ensuring reliable detection even at low concentrations. Validation results show excellent linearity, satisfactory intra- and inter-day precision, and high clinical correlation with conventional venous blood methods. These performances surpass those of immunoassays, particularly in the lower concentration ranges, which is crucial for women and children[2] . Testosterone is an androgenic hormone that plays an important role in both men and women, with circulating levels of total testosterone ranging from 1 to 1480 ng/dL. However, high-throughput immunoassays often lack precision in the lower concentration ranges (below 100 ng/dL), particularly when used in women or children, making this LC-MS/MS method all the more relevant to overcome this limitation [3]. A study by Wolken et al. demonstrated the method's validation, finding an intraday coefficient of variation (CV) of less than 10% and an interday CV of less than 15%. The assay had a limit of quantification of 0.5 ng/dL, with a range of analyte measurement from 2 to 1200 ng/dL [3] . Furthermore, a study by Xu, W., Li, H., Guan et al. confirmed the validity of the LC-MS/MS method, revealing excellent linearity for each analyte with a linear correlation coefficient greater than 0.992. Total accuracies at three concentrations were 15.66%, 8.81% and 4.34% respectively for testosterone. In addition, recovery, carryover, matrix effect and analytical specificity were evaluated, confirming the robustness of this method with satisfactory results. Finally, reference intervals (5th-95th percentile) were established for men and women [8].

II. Conclusion:

Although LC-MS/MS has many advantages, challenges remain, such as the need for specialized equipment and expertise, which may limit accessibility in some clinical settings. Nevertheless, its role in improving testosterone analysis is undeniable, paving the way for more reliable diagnostics and monitoring strategies.

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