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general introduction

based on ‘Testosterone assays: fitness for purpose’
Hong N. Bui, Marinus A. Blankenstein, Annemieke C. Heijboer

Annals of Clinical Biochemistry, provisionally accepted

Testosterone (17β-hydroxy-4-androsten-3-one, figure 1), the major androgen in men, is best known as a sex hormone responsible for the development and maintenance of the male phenotype. Primarily, testosterone has a key role in the sexual development and reproductive system in adults. In newborn boys, testosterone is responsible for the growth and differentiation of the genital tissue, such as the development of penis and prostate [1]. During puberty, growth and differentiation of the genital tissue continues and testosterone then takes up an important role in the regulation of hair growth, muscle mass, distribution of body fat and voice [2]. Non-sex-specific functions of testosterone include bone metabolism and bone re-modeling.

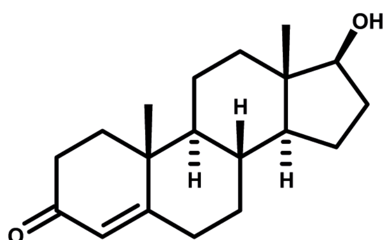

FIGURE 1: Chemical structure of testosterone. The steroid skeleton features a conjugated ketone function at the 3-position and a hydroxyl group at carbon 17.

Biosynthesis & regulation

The biosynthesis of steroids is similar in men and women [3]. The common precursor of steroid hormones is cholesterol. Testosterone can be synthesized via several pathways and the enzymes needed for each conversion are expressed in diverse quantities in different tissues (figure 2). In target tissue, testosterone is often converted to 5α-dihydrotestosterone (DHT), a more potent androgen. In addition, testosterone also serves as precursor to estrogens [4]. Even though androgen biosynthesis is chemically identical in both sexes, their production is organized in distinctive ways.

In *men*, 95% of total testosterone is produced in the Leydig cells, which are located in the testes. The remaining 5% relies on peripheral conversion and production by the adrenal glands. Testosterone production is regulated by the reproductive axis, which entails a complex ensemble of positive and negative feedback loops (figure 3). The hypothalamus secretes Gonadotrophin Releasing Hormone (GnRH), which has a positive effect on Luteinizing Hormone (LH) production in the pituitary. Under the influence of LH, testosterone production and secretion are increased [5]. Testosterone enhances sperm production. Meanwhile, testosterone exerts an inhibitory effect on the hypothalamus and LH production in men and women [6]. Figure 3 presents only a small part of a much more complex endocrine mechanism; much is still unknown about the interplay of actors in the reproductive axis and the fate of testosterone in target tissue.

In *women*, 25% of total testosterone is synthesized in the adrenal glands. Another 25% is produced in the ovaries under the influence of the hypothalamic-pituitary-gonadal axis , much in the same manner as the regulation in men. The remaining 50%

of testosterone is obtained by conversion of androstenedione in peripheral tissue [1]. Circulating testosterone levels in women are about 5-10% of those in men.

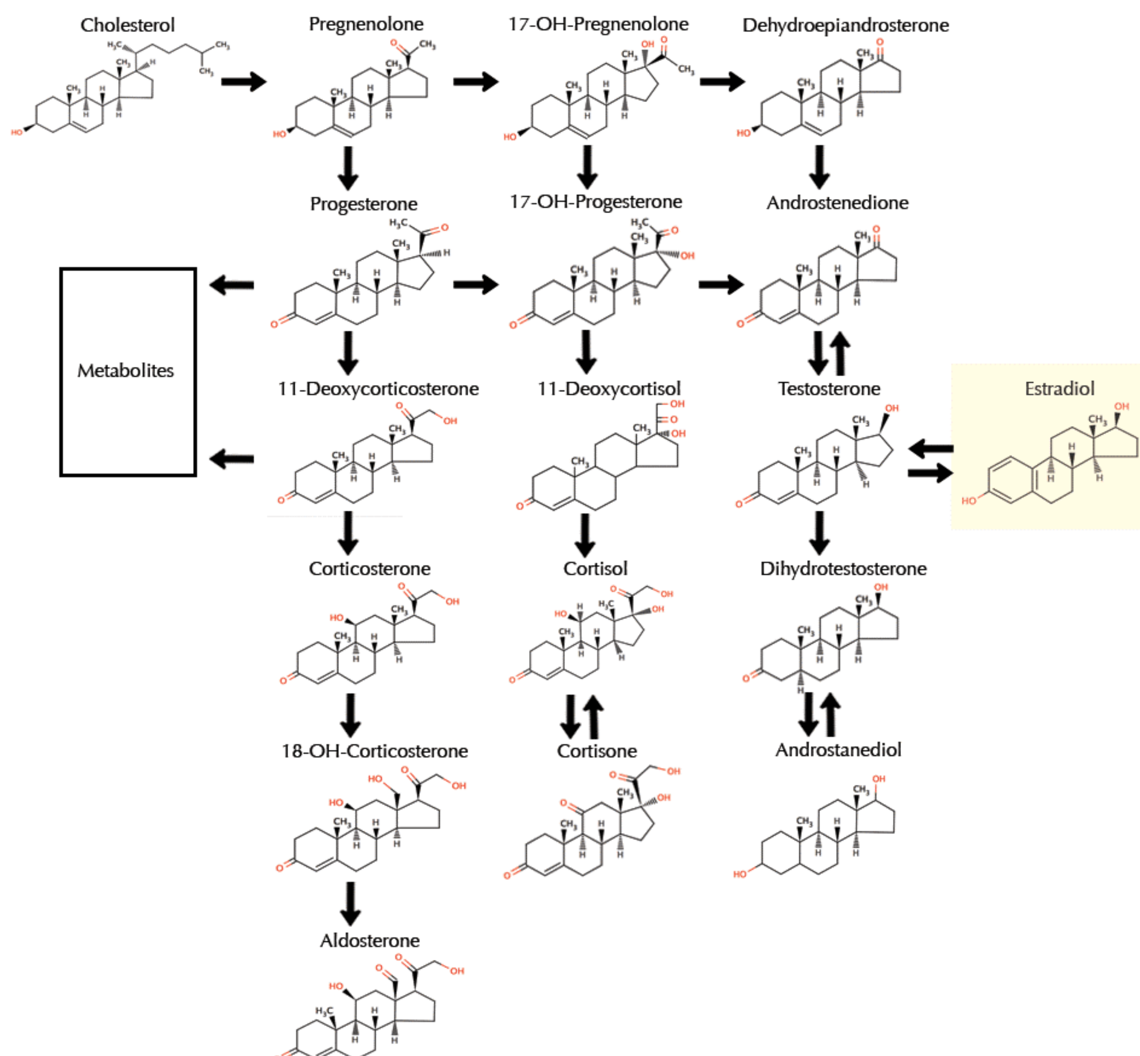

FIGURE 2: Steroid hormone biosynthesis. *Adapted from the Tulane/Xavier center for Bioenvironmental Research.*

Testosterone in the circulation

Regulation of circulating testosterone is likely to be more complex than described here, however, it is beyond the scope of this thesis to go into more detail [7]. Testosterone is, by the above-mentioned glands, released into the blood stream and transported in three different modes. The major part, 44–65% (in women 66–78%) is specifically bound to sex hormone-binding globulin (SHBG). SHBG is an α-globulin that has low capacity for steroids but binds with very high affinity ($K_a = 1 \times 10^8$ to 1×10^9). SHBG has the highest affinity for DHT and lowest for estradiol. The remaining part, 33–54% (in women 20–30%) is bound to non-specific proteins such as albumin; these proteins have a high capacity and a low affinity ($K_a = 1 \times 10^4$ to 1×10^6) for testosterone [8]. Only 2% of

testosterone circulates unbound. Testosterone may rapidly diffuse into target tissues such as skin and prostate, where it is often converted into DHT, the more potent androgen, by 5α-reductase [4].

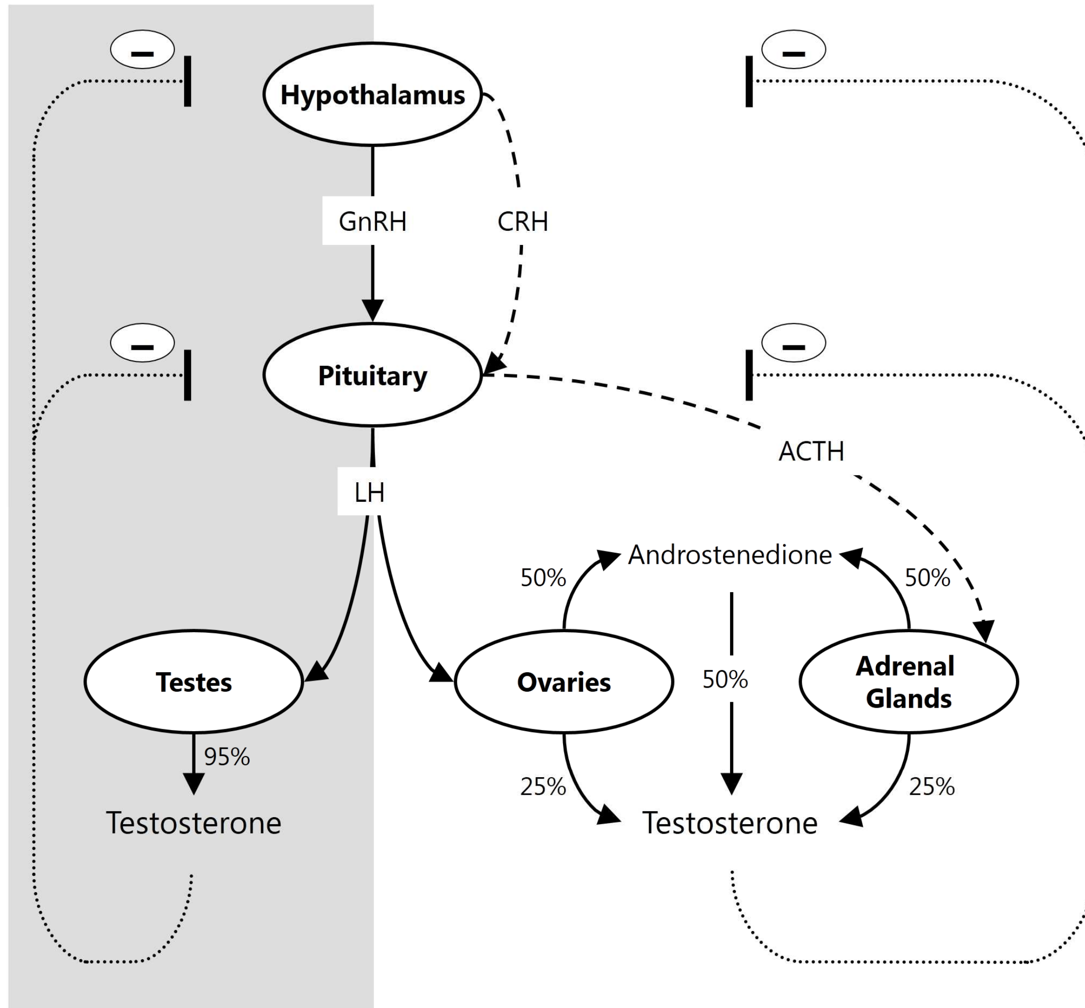

FIGURE 3: Schematic overview of the regulation of testosterone production in males (grey area, left side) and females (right side). In men and women, GnRH is secreted by the hypothalamus, which stimulates the pituitary to produce LH. Testosterone production and secretion by the testes and ovaries is increased under the influence of LH. In turn, testosterone exerts an inhibitory effect on the hypothalamus and LH production. In addition, testosterone is also acquired through peripheral conversion and in the adrenal glands, via CRH and ACTH (dashed lines). In women, a great amount of testosterone is produced through these two pathways. In men, these pathways only account for a small part of the total testosterone production. GnRH: Gonadotropin-releasing hormone, LH: luteinizing hormone, CRH: corticotropin-releasing hormone, ACTH: adrenocorticotropic hormone.

Serum total testosterone is the most frequently measured androgen in clinical practice. According to the free hormone hypothesis, free testosterone concentrations are more reflective of the physiologic actions as only free testosterone is able to diffuse into the

target tissue to bind to the androgen receptor [9]. Next to the free hormone hypothesis, it has been proposed that both free and albumin-bound testosterone (or non-SHBG bound testosterone) can be considered bio-available because testosterone can easily dissociate from albumin, due to the low affinity, and diffuse into target tissue [10]. The free androgen index, FAI, calculated as testosterone concentration divided by SHBG concentration has been proposed as a useful tool in specific diagnoses [11]. Until now, there is no consensus about the superiority of either of these indicators of the testosterone status: total testosterone, free testosterone (calculated or measured), bio-available testosterone, and free androgen index. Investigation has been hampered by inaccurate testosterone measurements due to analytical limitations [12]. Certain indicators of serum testosterone status have been thought to have more discriminative power than total testosterone in specific differential diagnosis. An excellent overview of the different parameters of serum testosterone has been written by Wheeler [13].

Clinical application

Measurement of testosterone in plasma or serum is an essential tool for the diagnosis, confirmation and monitoring of reproductive related disorders in both sexes. In men, the testosterone concentration together with gonadotropin levels, LH and FSH (follicle-stimulating hormone), gives insight into the origin of testicular dysfunction [14]. Androgen-deficiency due to primary testicular insufficiency, e.g. a defect in the Leydig-cells in the testes, will lead to hypergonadotrophic hypogonadism (LH↑ FSH↑ T↓) such as in Klinefelters-syndrome [15]. Androgen-deficiency due to secondary testicular insufficiency, e.g. as a result of a deficiency in the reproductive axis (pituitary and/or hypothalamus), may result in hypogonadotrophic hypogonadism (LH↓ FSH↓ T↓) [16]. Testosterone concentrations are also monitored to appreciate the effect of therapy in patients with prostate cancer [17]. Testosterone and other androgens are measured to confirm hyperandrogenism in women with or without clinical manifestations of hirsutism, acne and alopecia [18]. This androgen excess may be caused by PCOS or, less frequently, adrenal hyperplasia. Besides reproduction related disorders, high testosterone concentrations can be indicative of androgen-secreting tumors of ovarian or adrenal origin [19]. To facilitate sex assignment of newborns and young infants with ambiguous genitalia, testosterone measurement is involved. In these children, there is a so-called ‘window of opportunity’: until six months of age, boys produce testosterone [1;20]. After these six months, there is no detectable testosterone production until the onset of puberty, when the testosterone concentration will slowly rise. Furthermore, testosterone levels in children are assessed to determine pubertal stage, as well as during follow-up of children with precocious or delayed puberty [21;22]. Testosterone production may be induced temporarily by a human chorionic gonadotrophin (hCG)-test to confirm delayed puberty [23].

Testosterone measurement
In general, the techniques used for quantitative analysis of testosterone can be divided into two groups, competitive (radio-)immunoassays ((R)IA) and mass spectrometric methods (gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS)). Some general analytical challenges arise when measuring testosterone concentrations. First of all, concentrations can range over 3 orders of magnitude, depending on factors like gender, age, and the disease state [24]. Secondly, steroid hormones all have similar structures (figure 2) that may lead to cross-reactivity in some assays [25;26]. Thirdly, a universally calibrated standard has only become available since 2011, which implies that, until then, every laboratory or assay manufacturer had to set its own standard [12]. Despite the wide availability of highly purified testosterone and the relative ease with which standards can be prepared gravimetrically, this has turned out to be a virtually impossible task. Results of one sample, measured at different laboratories and/or by immunoassays from different manufacturers, can therefore show significant variation, which makes comparison between labs complicated [27].

Gas chromatography-mass spectrometry
Gas chromatography-mass spectrometry (GC-MS) is considered the gold standard for quantitative analysis of steroids. Gas chromatographic separation is highly reproducible and accurate [28]. In addition, the technique allows for measurement of multiple analytes in one assay. On the other hand, testosterone in serum/plasma needs to be released from its binding proteins and derivatization is required to improve volatility and chromatographic behavior. The losses during sample preparation are generally accounted for by the use of an internal standard, ideally a stable isotopically labeled counterpart of the compound that is being measured (isotope dilution (ID)). These assays typically require large sample volumes, up to 2 mL per replicate. Furthermore, GC-MS involves cumbersome sample preparation, it has limited throughput due to long analysis times, and handling of this technique requires highly trained personnel. Because of these disadvantages, GC-MS is not implemented in routine use. Currently, the Joint Committee of Traceability in Laboratory Medicine (JCTLM) has two published ID-GC-MS and one ID-LC-MS/MS procedure registered as reference methods for testosterone [28-32].

Immunoassays
Traditionally, the radioimmunoassay (RIA) for testosterone measurement involves solvent extraction and chromatographic purification to eliminate proteins and structurally related molecules. If thoroughly validated and properly executed, the accuracy of extraction-chromatography-RIA is comparable to ID-GC-MS and smaller sample volumes are needed to provide the same sensitivity. We owe much of our

knowledge of steroid hormone concentrations to these assays. When more specific antibodies became available, chromatographic separation was gradually abandoned. As fast throughput became more important, the liquid extraction step was eliminated as well (direct RIA), followed by the development of automated direct immunoassay, which are the most commonly used immunoassays in clinical practice. The omission of pre-immunoassay purification steps, however, has led to problems such as lack of specificity and precision. The performance offered by these automated immunoassay is at best acceptable for relatively high testosterone concentrations, which are characteristic of healthy men [33]. In the last decade, the quality of immunoassays, especially for measurement of testosterone in females and children, has been questioned [12;34]. Several studies have addressed the performance of direct immunoassays and politely concluded that there is a large range in quality – some immunoassays for measuring testosterone in women were 'no better than a guess' [27;33;35;36].

Although producing specific antibodies might be challenging, as steroid hormones are structurally similar, this is not necessarily the major cause of the poor performance. First, the incubation time in automated immunoassays to reach equilibrium is often relatively short, resulting in a larger influence of the matrix, which is sample specific and unpredictable. For instance, the antibody may bind to dehydroepiandrosterone-sulfate (DHEAS), which is present at high concentrations [25], but with a low affinity so it will easily dissociate. If the sample is measured before equilibrium is reached, DHEAS might still be attached to the antibody, resulting in cross reactivity. The performance of immunoassays might benefit from extended incubation times, but software packages for automated platforms generally do not include flexible options. Secondly, the majority of tracers used in direct immunoassays (e.g. acridinium, ruthenium complex, isoluminol) contain fairly bulky structural moieties in comparison to testosterone itself; as a result, the tracer may have significantly different properties than the native testosterone molecule and the antibodies can react differently towards endogenous testosterone than to the tracer. In RIAs, the radioactive iodine and especially the tritium tracers are only slightly larger than testosterone and incubation times are typically longer; hence, RIAs suffer less from these phenomena. Another factor that might influence the accuracy of direct immunoassays is the efficacy of releasing testosterone from its binding proteins in combination with the wide range of SHBG concentrations in different patient populations. Manual sample preparation, i.e. release testosterone from its binding protein and chromatographic separation, prior to immunoassay analysis is cumbersome and nowadays seldom used in clinical practice. In automated immunoassays testosterone is often displaced from its binding proteins by an overdose of a competitor for the binding sites that ideally has no affinity for the antibody, and/or by a pH shock. The completion of this procedure obviously is critical to the result of the assay.

The greatest advantage of immunoassays is that they are often automated and therefore feature high throughput. Another potential advantage is that a kit with reagent is bought from a manufacturer and ready to use. Direct automated immunoassays and radioimmunoassay kits include a tracer that is used to correct for procedural losses and to determine recovery of all sample preparation steps and the procedure incorporates to release testosterone from its endogenous binding protein.

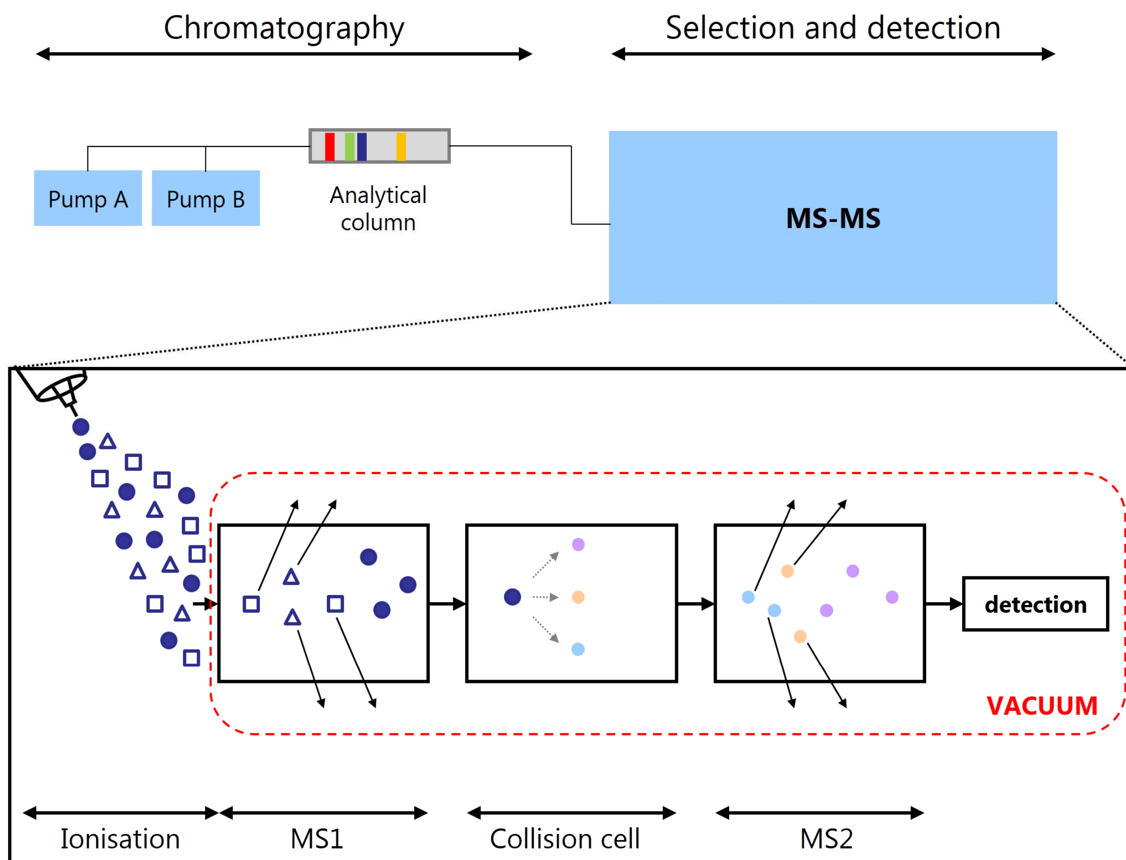

FIGURE 4: Schematic representation of the LC-MS/MS technique with electrospray ionization. By HPLC, the molecules in a sample are separated based on their affinity to the analytical column (stationary phase) and the fluid that is pumped through the column (mobile phase). When the separated molecules leave the analytical column in the mobile phase, the fluid enters the mass spectrometer through a needle. The fluid is evaporated under a stream of nitrogen and high temperature and the electric power on the needle ionizes the molecules. Then, the ionized molecules are sucked into the first MS chamber. The conditions in this chamber are set to select ions with a certain mass/charge ratio (*m/z*), only these ions are channeled through the collision cell where fragmentation takes place. The fragments that are specific to a compound of interest are selected by the second MS chamber and arrive at the detector.

Liquid chromatography-tandem mass spectrometry

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become more accessible in laboratory medicine in recent years. If adequately developed and validated, the LC-MS/MS has comparable accuracy, precision, sensitivity, and the possibility to do multiple analyte measurements similar to GC-MS [37]. A typical ID-LC-MS/MS procedure for measurement of testosterone includes: a stable isotopically labeled internal standard, a step to release testosterone from its binding protein, as with all testosterone assays, and depending on the type of instrument, derivatization may be necessary to increase sensitivity by enhancing ionization efficiency, or modifying the efficiency of the fragmentation pattern [38]. After this sample preparation, the sample is injected onto the LC-MS/MS. A schematic overview of this technique is depicted in figure 4. First, testosterone is separated from other molecules in the sample based on its affinity to an analytical column (LC). When testosterone leaves the analytical column, it enters the tandem-MS instrument where ionization takes place. Only the components with a certain mass/charge ratio *(m/z)* (parent-ion) are selected by the first MS and channeled through a so-called collision cell. In this cell, the selected components are fragmented and the fragments are selected by the second MS. This process results in a spectrum of smaller fragments that is unique for the component (daughter-ion). Thus, a defined 'daughter ion' from a defined 'parent ion' finally reaches the ion detector (figure 4). Chromatographic separation by LC has the advantage of shorter analysis times than GC and the detection of a confirmation ion-pair enhances specificity in LC-MS/MS. However, LC-MS/MS is a complex technique that like GC-MS, can only be carried out by specialized personnel.

AIMS AND OUTLINE OF THIS DISSERTATION

This dissertation aims to address the need for sensitive and accurate testosterone measurements. The Endocrine Society made a position statement and has appealed to the scientific community to improve the quality of testosterone measurements [12]. We have taken up this challenge and decided to develop a highly accurate and sensitive method for testosterone measurement and to evaluate its superiority over "conventional" methods with respect to its clinical applicability.

In **Chapter 2** we describe the development of such a highly sensitive and specific ID-LC-MS/MS including its analytical and clinical validation. This method was used to evaluate the accuracy of second generation immunoassays along with the most commonly used direct immunoassays in the Netherlands (**Chapter 3**). In addition, we investigated whether additional manual sample preparation could improve their performance.

Having established the method, we applied it to answer the following clinical questions:

- Is the concentration of testosterone in women dependent on the phase of the menstrual cycle?
- Are testosterone derived parameters like free testosterone or the free androgen index superior to the total testosterone concentration in the confirmation of the diagnosis of polycystic ovary syndrome?
- What is "the castration level" of testosterone in men on androgen deprivation treatment?
- Is it possible to perform serial testosterone measurements in saliva, in order to monitor androgen substitution?

The introduction of testosterone methods with improved accuracy, i.e. our ID-LC-MS/MS and 2nd generation immunoassays, at low concentrations (typically found in women) has enabled us to re-evaluate testosterone concentrations in women. We examined the daily dynamics of testosterone across the menstrual cycle and established reliable reference ranges, as described in **Chapter 4**. Furthermore, we examined the biological variation for testosterone and testosterone-derived parameters and assessed the diagnostic value of accurate testosterone measurement in the polycystic ovary syndrome, of which hyperandrogenemia is a key-feature. This is described in **Chapter 5**. Not only women can have low testosterone concentrations, also men with prostate cancer who are treated by androgen deprivation therapy have low testosterone concentration in their circulation.

In **Chapter 6**, we describe the evaluation of the efficacy of androgen deprivation therapies using our ID-LC-MS/MS method. The introduction of LC-MS/MS for sensitive and accurate testosterone analysis raises the question whether testosterone can also be measured in other matrices such as saliva that can be obtained non-invasviely and hence is more patient-friendly, but typically has a much lower testosterone concentration. This would be especially useful for sequential measurements. In **Chapter 7**, we therefore introduced an ID-LC-MS/MS method for testosterone in saliva and used this method to investigate the testosterone profiles after testosterone-ester mixture injections in female-to-male transsexual adolescents.

Finally, in **Chapter 8**, the results of the abovementioned studies are summarized and put into perspective and suggestions for the proper use of testosterone assays are presented.

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