1. Total testosterone assay is recommended as the first-line approach.

2. Radioimmunological assay following prior treatment of the sample (extraction or extraction + chromatography) is the recommended method pending wider experience with mass spectrometry.

3. Where testosterone is twice the upper limit of normal, it is recommended that DHEAS assay be performed. DHEAS is primarily of cortico-adrenal origin in women. Thus, a DHEAS level over 600 mg/dl indicates a diagnosis of androgen-secreting adrenal cortical adenoma. If DHEAS is normal, the diagnosis could be either ovarian hyperthecosis, normally associated with insulin resistance, or androgen-secreting ovarian tumour.

4. More rarely, elevated testosterone is associated with a marked elevation of SHBG possibly as the result of use of medication having an estrogenic effect (tamoxifen, raloxifene, Op’DDD), or of hyperthyroidism or liver disease.

5. Normal testosterone levels in patients with clear clinical symptoms of hyperandrogenism (hirsutism, seborrhoeic acne) must be interpreted with care. SHBG is normally reduced in the event of overweight, metabolic syndrome or familial history of diabetes.

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Keywords: Testosterone; Androgen measurement; Hyperandrogenism investigation

1. Question 1: Which androgen should be used to investigate hyperandrogenism in women?

Recommendation 1: Total testosterone assay is recommended as the first-line approach.

Comments:

- testosterone is the main circulating active androgen. Although it is recommended that it be measured in the early follicular phase, variations in testosterone levels throughout the female menstrual cycle are fairly insignificant. Testosterone levels have in fact been shown to correlate fairly well with severity of hirsutism;
- testosterone assay is essential for the diagnosis of androgen-secreting tumours.
3. Question 2: Which assay method should be used for testosterone?

**Recommendation 2:** Radioimmunological assay following prior treatment of the sample (extraction or extraction + chromatography) is the recommended method pending wider experience with mass spectrometry.

- The selected immunoassay kit should provide the best possible quality criteria (precision, low limit of detection, good correlation with the reference methods for low concentrations, use of an effective quality control technique).
- Testosterone assay should be performed using immunoassay preceded by purification (extraction or extraction followed by chromatography).
- Where this approach cannot be adopted, the direct immunoassay method must have the best possible quality criteria (precision, low limit of detection, good correlation with the reference methods for low concentrations, use of an effective quality control technique, etc.).
- Interpretation of results must be based on knowledge of the normal range of concentrations and the deviations seen in a population of normal women presenting no ovarian or metabolic disorders.

**Comments**

2.1. Immunoassay

Androgens are low molecular weight (around 300 g/mol) steroids that can be assayed using so-called competitive methods for assay of numerous analyses, including several androgens, among which is testosterone.

It is important to note that the identity of the matrix of control samples and the patients’ sera cannot be absolutely guaranteed, due to the actual lyophilisation process, amongst other things. Bearing in mind the foregoing limitation, analysis of results obtained over time has prompted a number of observations.

The vast majority (over 95%) of laboratories carrying out total testosterone assay (more than 300 laboratories) use direct methods without prior extraction before immunoassay.

There is considerable *dispersal of all values* for control sera exhibiting a concentration close to that found in women (Fig. 1); this variation is due to differences between the assay kits themselves as well as the lack of precision of most assay kits at these concentration levels. Dispersal of values decreases as the concentration of control samples increases and becomes acceptable for samples containing concentrations comparable with those seen in men (Fig. 2).

Most immunoassays yield values lower than GC-MS (Fig. 2) but concordance between the two methods is also dependent on concentration and presents less heterogeneity being seen in concentrations determined for men.

**In summary,** recent observations from external evaluation of the quality of total testosterone assays support the conclusions of a study performed in 2001 by the French Society of Clinical Biology (see reference in Fig. 1 legend) concerning evaluation of the analytical performance of 10 types of direct immunoassay (eight automatic analysers and two RIA methods) and which produced the following recommendations:

- direct assay kits should be used for laboratory investigation of untreated men;
- they should not be used in specific disease settings such as the investigation of pubertal disorders, hyperandrogenism in women or monitoring of hormone replacement therapy, because of the observed differences in accuracy.
in which steroids in the unknown sample compete with a known quantity of labelled steroids to bind to a defined and limited number of antibody binding sites. When equilibrium is reached, the steroid-antibody reaction may be quantified by separating free steroid (fraction F) from antibody-bound steroid (fraction B) and then measuring the signal emitted by fraction B [1]. With this type of method, the quantity of steroids to be assayed is inversely proportional to the Ab-labeled steroid. The calibration curves (which represent the signal from the fraction of steroids bound to the antibody as a function of the concentration of steroid to be assayed) are thus decreasing. The chief markers used are: radioactive markers (iodine 125 and tritium), enzymatic markers (HRP and PAL), fluorescent markers (umbelliferone, europium chelates), and chemiluminescent markers (luminol, acridinium esters, dioxetanes).

2.1.1.1.1. The advantages of direct immunoassay.  
Two different immunoassay methods exist for T and Δ4:

2.1.1.1. Direct immunoassay. Immunoassay is performed directly on the patient’s plasma or serum, in most cases using an automatic immunoanalysis device (with nonradioactive markers) or else using kits (with radioactive markers).

2.1.1.1.2. The disadvantages.  
They relate to the absence of prior treatment of the biological sample, and thus to the potential presence of interference and matrix effects, despite good specificity of the antibodies used. These effects, which can lead to erroneous results above or below the actual level, are seen primarily when low concentrations of steroid are being assayed, as is the case with testosterone assay for women [2–6].

2.1.1.2. Immunoassay with prior treatment of the biological sample. Steroid extraction using an organic solvent prior to immunoassay eliminates much of the matrix and, in particular, transport proteins (albumin and SHBG).

Following extraction, purification of the extract by chromatography (celite, HPLC, Sephadex LH20) eliminates steroids likely to interfere with the immunological reaction because of similar structure to the steroid being assayed and insufficient antibody specificity (e.g., 5α-DHT, Δ4 and Δ5-androstenediol for testosterone assay) [3,7,8].

2.1.1.2.1. The advantage of immunoassay with prior biological sample treatment.  
It is thus of high specificity. The values found are in fact very close to true values as indicated by the good correlation between these values and those obtained with mass spectrometry.

2.1.1.2.2. Disadvantage.  
The disadvantage of these methods is their impracticality (manual methods, long analysis time, need for specialist laboratories, generally laboratories working with radioelements).
In summary, direct immunoassays of testosterone and Δ4 (which today are automated in most cases) are generally reliable. However, results must be interpreted carefully since reference values may vary between methods, and thus from one laboratory to another. Discordant, or even erroneous values, can occur because of adverse matrix effects. Immunoassay incorporating prior sample treatment is extremely reliable with good accuracy since matrix effects are eliminated.

2.1.2. Immunoassay of dehydroepiandrosterone sulphate (DHAS)

Since DHAS is a water-soluble antigen present in human plasma in large quantities (around 6 μmol/L in young subjects), immunoassay is performed directly on plasma with or without dilution, depending on the method used (radioimmunoassay, automated immunoassay with a nonradioactive marker, etc). Use of highly specific antibodies renders interference from other steroids highly unlikely given the concentration level of this androgen [5].

2.2. Androgen assay by mass spectrometry

The gold standard for androgen assay has long been gas chromatography coupled with mass spectrometry (GC-MS) [9,10]. This method of choice allows simultaneous quantitation of several androgens. However, while this technique requires incorporation of the analytes in the gas phase, the majority of these substances are nonvolatile and derivatisation is thus necessary to alter their chemical structure and vaporise them. Commonly used derivatisation agents include trimethylsilyl (TMS), tert-butyldimethylsilyl ether (BDMS), heptafluorobutyric ether (HFB) and hydroxylamine [11,12]. This method, which produces values close to true values (i.e. unbiased by interfering compounds), requires lengthy preparation (extraction, derivatisation) as well as large quantities of sample for assay and it is therefore too complex for day-to-day use.

For a number of years, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has widely rivalled GC-MS. The emergence of atmospheric pressure chemical ionisation (APCI) and of electrospray ionisation (ESI) has allowed direct coupling of liquid chromatography and mass spectrometry (LC-APCI and LC-ESI, respectively) [6,13–16]. LC-MS/MS has a considerable advantage: since the eluent phase is a liquid, derivatisation is unnecessary in most instances. This is the case for testosterone, which possesses unsaturated carbonyls α and β that are readily ionised without derivatisation. However, since 5α-DHT is a neutral steroid that is difficult to ionise using standard ESI or APCI ionisation methods, derivatisation is necessary in order to obtain a sufficiently low level of detection [17].

It has recently been shown that the use of extremely high pressures, far higher than with standard LC, produces unrivalled performance in terms of separation [18,19]. This approach forms the basis for the development of ultra performance liquid chromatography (UPLC), which follows the principles of standard HPLC but with improved analysis speed, sensitivity and resolution.

In summary, mass spectrometry (MS) is the absolute gold standard for assay of steroids and of androgens in particular. It is a precise and accurate method and its limits of detection are sufficiently low for assay of the generally small quantities of androgens present in the sera of women (and children).

MS (GC-MS or LC-MS) is not widely used at the moment due to:

- costs of materials (too high for most laboratories);
- the special skills required;
- lack of convenience (complicated techniques, impossibility of performing large series of assays).

However, in the not-too-distant future, LC-MS/MS could be suitable for routine use and provide an alternative to immunoassay when automatic analysis becomes possible.

Fig. 3. Decision tree for total testosterone tumoral elevation: twice the upper limit of normal, or greater than 100 ng/dl (3.5 nmol) (assay with extraction) or 200 ng/dl (7.0 nmol) (direct assay). * 16000 nmol/l. ** 30.3 nmol/l.

Arbre décisionnel devant une élévation tumoriale de la testostérone totale : deux fois plus élevée que la valeur supérieure de la normale, ou supérieure à 100 ng/dl (3,5 nmol) (dosage avec extraction) ou 200 ng/dl (7,0 nmol) (dosage direct). * 16000 nmol/l. ** 30,3 nmol/l.
3. Question 3: What diagnostic procedures should be favoured for elevated testosterone levels?

Recommendation 3: (see decision tree, Fig. 3).

“In a patient with a testosterone level twice the upper limit of the normal”, it is recommended that DHEAS assay be performed. DHEAS is primarily of corticoadrenal origin in women. Thus, a DHEAS level of over 600 μg/dl (16000 nmol/l) indicates a diagnosis of androgen-secreting adrenal cortical adenoma (often associated with hypercorticism) and an abdominal scan must be performed rapidly [20–22]. If DHEAS is normal, the diagnosis could be either ovarian hyperthecosis, normally associated with insulin resistance, or androgen-secreting ovarian tumour. In both cases, elevation in testosterone is LH-dependent and is restricted by GnRH agonist, oestrogen-progestogen or cyproterone acetate [23].

More rarely, elevated testosterone is associated with a marked elevation of SHBG possibly as the result of use of medication having an estrogenic effect (tamoxifen, raloxifene, Op’DDD), or of hyperthyroidism or liver disease, particularly portal hypertension with primary cirrhosis.

Where testosterone is just above the normal upper limit, the most likely diagnosis is polycystic ovary syndrome (PCOS). However, screening should be performed for the nonclassic form of 21-hydroxylase deficiency (assay of 17OHP) and depending on the clinical setting, Cushing disease must be ruled out.

4. Question 4: What diagnostic approach should be adopted for normal testosterone levels?

Recommendation 4: Normal testosterone levels in patients with clear clinical symptoms of hyperandrogenism (hirsutism, seborrhoeic acne) must be interpreted with care.

Assay conditions must be carefully weighed, with checking of the period of the cycle based on the return of menstruation following the assay date, absence of treatment with thyroid hormones (or analogues such as Triacana®) or with drugs with estrogenic effects, which could increase the concentration of the transport binding protein SHBG (e.g. antiepileptic agents such as Depakine®) [24].

If there is any suspicion of PCOS (irregular cycles with pronounced hirsutism), it is recommended that assay be repeated or an optional assay be selected:

- SHBG is normally reduced in the event of overweight, metabolic syndrome or familiar history of diabetes. It can be used to correct interpretation of total testosterone and allows free or non-SHBG bound testosterone to be determined [8];
- Δ4-androstenedione has not been studied comparatively with testosterone, but dissociations exist, with isolated elevation of Δ4-androstenedione but no elevation of testosterone, particularly in the event of reduced SHBG;
- 17OHP to avoid overlooking the nonclassic form of 21-hydroxylase deficiency (see recommendations concerning diagnosis).

Fig. 4 shows a decision tree recommended for use with elevated tumoural total testosterone.

References


