Androgen assessment in hirsutism and alopecia

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■ Advances in technology, such as saturation analysis and nonisotopic immunoassays, have improved the measurement of androgens, binding proteins, and hormone receptors, and clarified the diagnosis of idiopathic hirsutism. Immunoassay methods in steroid biochemistry enable accurate measurement of low concentrations of various androgens and their metabolites; and high-specificity antisera with chemical blocking agents allow measurement of these steroids directly in plasma without resorting to extraction methods. The future may bring the capacity to measure free concentrations.

□ INDEX TERMS: ANDROGEN; HIRSUTISM □ CLEVE CLIN J MED 1990; 57:292–297

RESEARCH in recent years favors the hypothesis that patients with idiopathic hirsutism have increased androgenic activity either at the peripheral level or at a localized level in target tissues.[1] There are three different pathways of androgen excess: (1) increased androgen production at the glandular level; (2) alteration in androgen-binding proteins that may increase the concentrations of free steroids; and (3) target organ abnormalities, such as sensitivity to androgens or the ability of involved skin to convert these steroids to more active metabolites.

Concurrent with research into the pathogenesis of hirsutism, advances in laboratory technology have improved the measurement of androgens, binding proteins, and hormone receptors, and helped in the documentation of the three pathways noted above. The technology responsible for these achievements is saturation analysis, including radioimmunoassay (RIA). RIA has been the method of choice for measuring these steroids, primarily because of its unique potential for quantifying these hormones in unextracted serum.

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TECHNICAL CONSIDERATIONS

Steroid radioimmunoassays present several problems. Most steroids are not immunogenic, and they are all similar in structure; ie, they all have the same cyclopentaphenanthrene nucleus with only minor structural variations. Consequently, it is difficult to generate specific antibodies. A steroid molecule can be made immunogenic by coupling it chemically to a carrier protein. Such coupled steroid to protein is known as a "hapten"; antibodies can then be raised in rabbits or other animals by immunization with haptens.[2] The site of the molecule where the protein is conjugated has a significant impact on the specificity of the resulting antibody.

Figure 1 shows the structural formulas of three androgenic steroid hormones and of deoxycorticosterone, progesterone, and 17-beta estradiol. These steroid hormones show only minor structural differences; haptens made by coupling bovine serum albumin (BSA) to three

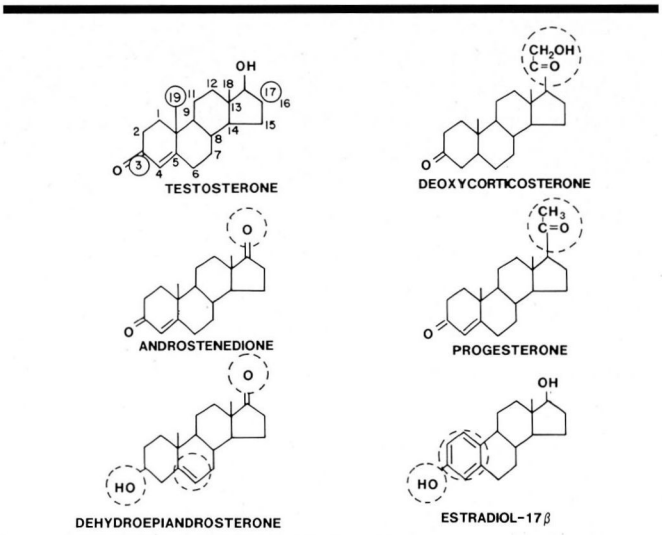

FIGURE 1. Structural formulas for three androgens and for deoxycorticosterone, progesterone, and 17β estradiol. The carbon positions circled in testosterone (3, 17, and 19) are used to couple carrier protein (BSA) for immunization protocols. The dotted circles in other molecules show the areas that differ in structure from testosterone.

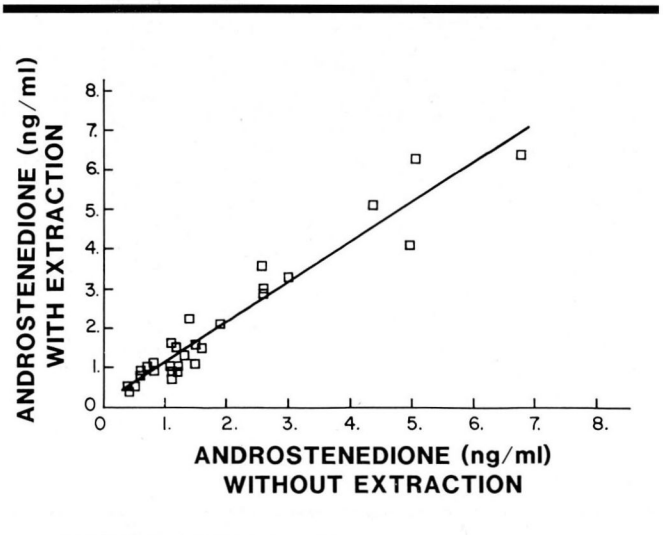

FIGURE 2. Comparison of direct androstenedione assay (no extraction step) with an assay that requires extraction of serum with anhydrous ethyl ether. Correlation coefficient (r) 0.96 ($P<.0001$).

different positions of carbon atom on the testosterone molecule show different specificities (*Table 1*). Antibodies raised by 3-BSA hapten will cross-react only with dihydrotestosterone (DHT). In contrast, antibodies made to 17-rabbit serum albumin show significant cross-reaction with androstenediol, progesterone, and deoxycorticosterone.[2] This assay system shows even more specificity with antibodies raised to conjugates of BSA coupled at the 19th position.[3]

For clinical interpretation, it is important to know cross reactivity data on each antibody selected for a given assay. Although most commercial reagent companies provide that data, the information is not always reliable and we recommend that the laboratory check cross-reactivity.[4]

Binding proteins in the serum bind some of these androgens with fairly high affinity and compete with antibody; this can interfere with the measurement of steroid molecules by RIA, make direct serum measurements difficult, and necessitate an extraction procedure with organic solvents. Certain chemicals, such as 8-anilinonaphthelene sulfonic acid, can inhibit binding of these steroids to proteins without affecting antibody binding and allows direct measurement of the steroid hormones. *Figure 2* compares the results of a direct assay with an extraction method for measuring androstenedione. Elimination of the extraction step simplifies these assays and results in a more precise measurement. Today, total testosterone, androstenedione, and dehydroepiandrosterone sulfate (DHEAS) are measured by such specific and precise immunoassays in most clinical laboratories.

TABLE 1
CROSS-REACTIVITY OF TESTOSTERONE ANTIBODIES

Steroid	% Cross-reactivity		
	Testosterone-3*	Testosterone-17*	Testosterone-19†
Testosterone	100	100	100
Dihydrotestosterone	22	–	3.5
Androstenedione	0.2	31	0.5
DHEA	0.05	<.01	<.01
Deoxycorticosterone	0.1	55	<.01
Estradiol 17ß	0.04	<.01	<.01
Progesterone	0.04	55	<.01

*Abstracted from reference 3
†Currently used in our laboratory

MEASUREMENT OF ANDROGENS

The adrenal androgens are biologically weak but they are converted in many tissues of the body to more potent

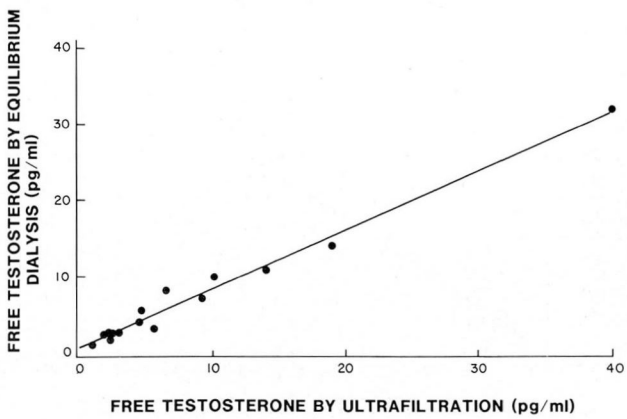

FIGURE 3. Comparison of free testosterone levels as measured by equilibrium dialysis with ultrafiltration assay. Correlation coefficient (r) = 0.99 ($P<.0001$).

androgens such as testosterone. The androgenic activity of androstenedione is about 15% that of testosterone, and dehydroepiandrosterone (DHEA) has about 8% of the androgenic activity of testosterone. Androstenedione and DHEA are secreted as C19 steroids and excreted into the urine primarily as sulfates. They are measured as the urinary 17-ketosteroids by Zimmermann reaction—a widely used measure of adrenal androgens. The 17-ketosteroids represent a mixture of nonspecific chromogens and steroid metabolites,[5,6] the major contributors being DHEA and DHEAS. Testosterone and DHT contribute less than 1%.[7] Although DHEAS is a weak androgen, it is present in serum in high concentrations (almost 1,000-fold greater than DHEA). Therefore, the plasma level of DHEAS is a better index of adrenal androgen secretion than 17-ketosteroids and, in fact, has replaced the latter in most clinical laboratories.[8,9]

MEASUREMENT OF DHEAS

The large pool of circulating DHEAS has a slow turnover rate; this, combined with little or no diurnal variations makes its measurement a reliable and simple indication of adrenal androgen activity. In recent years, simple and direct RIAs have been developed to determine DHEAS and are commercially available. Most of these assays are competitive RIAs that use immunologic separation techniques. A solid-phase, coated-tube method is simpler to perform, but showed nonlinear responses when serum samples were diluted serially.

Unlike cortisol, DHEAS does not exhibit significant diurnal variations, is not influenced by alterations in binding proteins, and shows excellent day-to-day stability. However, plasma levels of DHEAS show age variations that increase steadily from about the seventh year of life then gradually decline after the third decade.[10]

MEASUREMENT OF ANDROSTENEDIONE

Androstenedione is produced in both the adrenal glands and the ovaries. Published RIA procedures use antibodies raised against androstenedione derivatives attached to a carrier protein through positions 3, 6, 11, or 19.[11–13] Interference with cross-reactive testosterone has been the problem with these assays. This interference has been overcome by use of specific antisera to testosterone in the assay.[13]

With new commercial procedures, direct measurement of androstenedione without extraction is possible (*Figure 2*).

MEASUREMENT OF TESTOSTERONE, FREE TESTOSTERONE

Assays for measurement of total testosterone have been used since the early 1970s. The earlier assays required extensive extraction and purification procedures because the antisera had limited specificity. In recent years more specific antibodies have become available and, with the use of binding protein inhibitors, it has become possible to measure total testosterone directly in unextracted serum.

Total testosterone in hirsute women overlaps significantly with levels seen in normal women; in our experience, very few patients have values above normal range. Measurement of free fraction of testosterone correlates better with disease,[14] but direct measurement of free testosterone has been impossible because of its very low concentrations. It has been measured by equilibrium dialysis, a time-consuming and difficult technique for most clinical laboratories.

In recent years, an ultrafiltration technique has been used instead,[15,16] which depends on MPS-1 centrifugal gel filtration devices. This gel filtration assay correlates well with equilibrium dialysis (*Figure 3*).

MEASUREMENT OF ANDROGEN-BINDING PROTEINS

Another significant factor that affects and can modulate free androgen levels is sex hormone-binding

globulin (SHBG), also known as sex steroid-binding globulin (SSBG).[17] Like thyroid hormones, testosterone circulates largely bound to SHBG. A small portion of tes-tosterone, known as non-SHBG bound testosterone, is also weakly bound to albumin or to proteins like cortisol-binding globulin (CBG).

SHBG, a beta globulin, has been purified and studied extensively. Thyroid hormones increase its synthesis and release by the liver.[18,19] Approximately 98% of testosterone and about 30% of estradiol circulate bound to SHBG. DHT has the highest binding affinity for this protein when compared to other steroids. When measured at 4°C, the dissociation constant for DHT is approximately 0.4×10^{-9} M; for testosterone, 0.9×10^{-9} M; and for estradiol, 4.8×10^{-9} M.[20] At physiologic temperatures, DHT has almost no binding to other proteins like CBG or albumin. Testosterone, on the other hand, also binds with low affinity (kd in the range of 10^{-5} M) to albumin. This difference in binding affinity allows use of ^{3}DHT as ligand in assessing SHBG concentrations and use of ^{3}H-testosterone as ligand for assessment of non-SHBG bound testosterone.[21]

The binding of testosterone in female plasma to SHBG differs from the binding of thyroid hormones to thyroid hormone-binding globulin. Thyroid hormone levels are controlled primarily by the hypothalamic pituitary thyroid axis, and the adrenal gland is regulated by ACTH via a cortisol feedback loop. There is no feedback loop in female plasma for testosterone and, therefore, no known mechanism to return the concentration of free testosterone to the normal range when the plasma concentration of SHBG is altered. Therefore, measurement of these binding proteins is critical for assessment of free hormone concentrations of androgens.

MEASUREMENT OF SHBG

SHBG measurement involves saturation analysis and the use of Scatchard plots,[22–24] which give a rapid and inexpensive estimate. A time-resolved fluoroimmunoassay for direct measurement of SHBG has recently become available, and the two assays correlate closely (*Figure 4*).

Direct measurement of SHBG is valuable in the assessment of certain conditions such as cirrhosis and thyrotoxicosis, but it has limited use in the evaluation of patients with hirsutism, who often have SHBG levels in the normal range. When total testosterone concentrations are known, it is possible to assess the concentrations of unbound (free) testosterone by adjusting plasma testosterone levels on SHBG concentrations. The ratio of testosterone (ng/dL) to SHBG (ng/dL) is a better discriminator of hirsutism than either total testosterone or SHBG levels.[25,26]

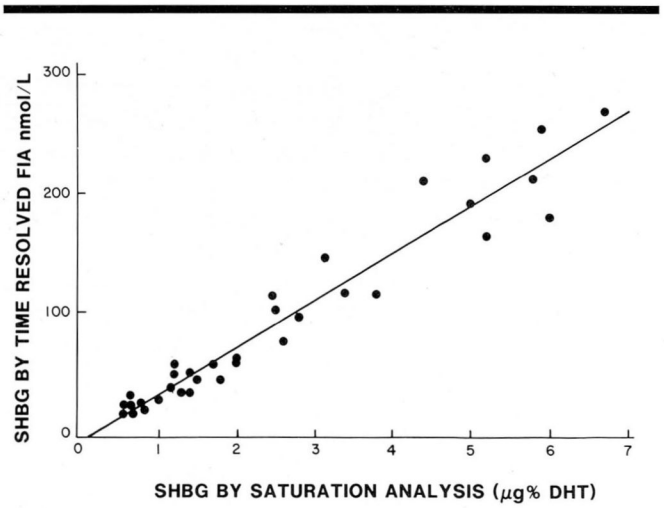

FIGURE 4. Comparison of SHBG levels as measured by saturation analysis and time-resolved fluoroimmunoassay. Correlation coefficient (r) = 0.96 (P<.0001).

Testosterone is also bound to albumin and, to some extent, to CBG. The binding is of low affinity with a rapid dissociation time compared to SHBG, suggesting that this loosely bound androgen may be biologically available through dissociation during capillary transit. Cumming and Wall[27] provided evidence for this hypothesis and suggested that non-SHBG bound testosterone may be a marker to identify hyperandrogenism in hirsute women. The measurement of non-SHBG binding is based on differential precipitation of plasma proteins by ammonium sulfate following equilibration of the plasma sample with tracer amounts of ^{3}H testosterone.

ANDROGENS IN SALIVA

Measurement of androgens in saliva has attracted considerable attention in recent years.[28] SHBG is either undetectable or minimally present in saliva. Therefore, measurement of steroids in this biological fluid reflects the free fraction of plasma steroids.[29] The ease and noninvasive collection of saliva combined with the simplicity of steroid measurement (as compared to free steroid assays in plasma) makes salivary steroid measurement a promising and attractive alternative to plasma steroids. However, there are discrepancies in the reported ranges of normal, which may delay the routine use of this technique in clinical laboratories.[30,31]

ASSESSING TARGET ORGAN ABNORMALITY

Weak androgens like androstenedione and testosterone are converted to more active metabolites—eg, DHT in skin—by 5-alpha reductase activity.[32] An increase in this enzyme activity may be associated with increased production of DHT, which is known to have more androgenic activity than the parent compound testosterone or its other precursors. DHT binds to the specific receptor protein with higher affinity than testosterone itself. These findings favored the hypothesis that an increased sensitivity of the skin to androgens is responsible for androgen excess in hirsutism in the presence of normal plasma androgen levels. However, attempts to demonstrate alterations in androgen receptor concentrations in human skin cytosol show no differences in receptor binding between men, women, and hirsute women.[33]

DHT is further metabolized to androstenediol glucuronide (3-alpha diol) or 3-beta androstenediols. Measurement of 3-alpha diol is a specific and sensitive marker of hirsutism.[34] A radioimmunoassay for 3-alpha diol has been developed for precise quantitation of this metabolite in serum,[34] but the reagents are not yet commercially available. Measurement of plasma levels of DHT have not proven useful in the diagnosis of hirsutism.[35] But recently developed methods to measure the 5-alpha reductase activity in skin have been found very useful in that regard.[36] Serafini and associates described a simplified, rapid, highly reproducible technique to measure 5-alpha reductase activity in small biopsy samples from involved skin;[37] the mean conversion ratio of testosterone to DHT in 23 normal women was 4.48 ± 0.36%, compared to 16.8 ± 1.6% in 20 hirsute women ($P<.01$). Their data suggested that measurement of DHT formation is best suited for assessment of 5-alpha reductase activity, and determination of 5-alpha reductase activity in vitro from a small genital skin biopsy specimen is suitable for the clinical evaluation of hirsutism. Although this technique has diagnostic value, it is cumbersome and requires technical expertise; these factors may limit its routine use in clinical laboratories.

CLINICAL RELEVANCE

Today's assay techniques allow precise measurement of most androgens in plasma; this in turn permits detection of low levels of these steroids in normal women and of subtle increases seen in idiopathic hirsutism. When the circulating level of only one androgen is increased, that analyte is usually DHEAS. We have found elevated DHEAS levels in almost 50% of patients with idiopathic hirsutism. The degree of elevation of DHEAS in these patients is modest, ranging from 3,000 µg/L to 6,000 µg/L. Increased DHEAS levels implicate the adrenal gland as a cause of hyperandrogenism, and levels greater than 8,000 µg/L suggest an adrenal tumor. Elevated androstenedione is associated less frequently with idiopathic hirsutism (18%) and usually occurs in the same patients who demonstrate DHEAS elevation. Elevation of free testosterone or free androgen index (total testosterone/SHBG) is seen in 10% of patients with normal DHEA levels; thus, androgen abnormalities are present in almost 60% of patients with idiopathic hirsutism, and at least one third of patients with clear evidence of hirsutism may have normal androgen levels. These individuals may have abnormal target tissue response and require further workup with assessment of 5-alpha reductase activity.

Gonadotropin, or luteinizing hormone (LH) and follicle-stimulating hormone (FSH), levels are particularly useful in the differential diagnosis of hirsutism. In hirsute women with polycystic ovarian disease, LH levels are elevated and FSH levels are low-normal; an LH/FSH ratio of three or greater is considered diagnostic for this condition and a ratio of 2:1 or greater is highly suggestive.

CONCLUSIONS

Immunoassay methods in steroid biochemistry have initiated a quantum leap in our ability to measure the low concentrations of various androgens and their metabolites. High-specificity antisera with chemical blocking agents allow measurement of these steroids directly in plasma without extraction. The future may bring even more sensitive immunoassays that involve nonisotopic technology and monoclonal antibodies capable of measuring free concentrations. The recent development and commercial availability of nonisotopic immunoassays for the measurement of SHBG should increase the clinical utility of this test. Although measurement of androgens and their binding proteins is simple and routine in most clinical laboratories, the assessment of target organ abnormality (testosterone to DHT conversion ratio) remains sophisticated and is performed only in research laboratories.

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