Radioimmunoassay of Plasma Testosterone, with Use of Polyethylene Glycol to Separate Antibody-Bound and Free Hormone

Peter H. Anderson,¹ Kimiko Fukushima, and Harvey S. Schiller²

We have developed a reliable radioimmunoassay for testosterone in plasma, polyethylene glycol ("Carbowax 6000") being used to separate antibody-bound and free hormone. Testosterone is separated from interfering steroids, notably dehydrotestosterone, by liquid–liquid partition chromatography on infusorial earth (Celite). The assay is sensitive (9 pg/liter), precise, and accurate. The lowest measurable concentration of testosterone is 350 ng/liter for plasma from men and 70 ng/liter for plasma from women. Intra- and inter-assay coefficients of variation were 6.9% and 9.7%, respectively, for plasma from men, and 9.6% and 11.8%, respectively, for plasma from women. Our method for separating antibody-bound and free hormone is practical and convenient and may be generally applicable to all radioimmunoassays of steroid hormones in plasma.

Additional Keyphrases: improved radioimmunoassay of steroids • normal values • sources of analytical error

Several methods for measuring plasma testosterone³ have been described, including the cumbersome double-isotope derivative technique (1–3) and gas-liquid chromatography (4, 5). The development of competitive protein binding assays (6–9) in which sex steroid-binding plasma protein is used and, more recently, sensitive radioimmunoassays (10–17) have permitted the measurement of testosterone in a large number of samples at the same time.

Because of the greater sensitivity and specificity achievable with commonly available high-affinity antisera, radioimmunoassay has become the method of choice for measuring plasma testosterone. The specificity is generally improved by a preliminary purification of the sample by some form of chromatography. Here, we demonstrate how an infusorial earth (Celite) microcolumn can be so used in a testosterone radioimmunoassay.

Separation of antibody-bound and free steroid is a critical part of all radioimmunoassay procedures. Various methods—including ammonium sulfate precipitation (10), dextran-coated charcoal adsorption (11–17), and the double antibody technique (18)—have been used for radioimmunoassays of testosterone or 17β-ol androgens. This is the first report of a reliable, specific radioimmunoassay for plasma testosterone with use of polyethylene glycol to separate antibody-bound and free hormone.

Materials and Methods

Materials

Antisera: A lyophilized antisera (S-741 No. 2; Professional Staff Association, Harbor General Hospital, Los Angeles, Calif. 90502) was reconstituted in distilled water and small aliquots were frozen at −70 °C until use, when it was diluted with the buffer to achieve an initial binding of 60–70% (usually 350-fold dilution).

Buffers: The stock sodium barbital–acetate buffer (pH 7.4) was prepared by dissolving 7.357 g of sodium barbital and 4.857 g of anhydrous sodium acetate in 250 ml of distilled water. The working barbital–acetate buffer, used for dissolving bovine γ-globulin and polyethylene glycol (see below), was prepared by mixing 50 ml of the stock solution with 900 ml of sodium chloride solution (8.5 g of NaCl per liter) and

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⁴ Trivial names and abbreviations used: testosterone, 17β-hydroxy-4-androsten-3-one; dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; androstenedione, 4-androsten-3,17-dione; androstenediol, 5-androsten-3β,17β-diol; cortisol, 11β,17,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5α-androstan-17-one; estradiol-17β, 1,3,5(10)-estratriene-3,17β,18β-triol; estrone, 3-hydroxy-1,3,5(10)-estratrien-17-one; progesterone, 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; PEG, polyethylene glycol.
⁵ Received Nov. 25, 1974; accepted Jan. 28, 1975.
adjusting the pH with hydrochloric acid (6 mol/liter) to pH 7.4. The working barbital–acetate–gelatin buffer, used in all other instances, was prepared similarly except that 1 g of reagent-grade gelatin (Mallinckrodt Chemical Works, St. Louis, Mo. 63160) was added per liter.

Steroids and testosterone standards: Testosterone and dihydrotestosterone were obtained from Sigma Chemical Co., St. Louis, Mo. 63178, and their purity was assessed from their melting points. A stock standard of testosterone (1 mg/ml) was prepared in ethanol every six months. From this, a working standard of 1 ng/ml was prepared every three to four weeks by diluting the stock standard with the buffer. Nonradioactive steroids were obtained from Sigma Chemical Co., and Steraloids, Inc., Pawling, N. Y. 12564.

\[1,2-^3H\text{Testosterone}\text{ (spec. activity 45 Ci/mmole)}\] and \([6,7-^3H]\text{dihydrotestosterone}\text{ (spec. activity 44 Ci/mmole)}\) were obtained from New England Nuclear Corp., Boston, Mass. 02118. Their purities were tested every three to six months by thin-layer chromatography, and they were used as long as their purity was greater than 95%.

Solvents: Ethyl acetate (Mallinckrodt) was glass-distilled before use. Absolute ethanol (U.S. Industrial Chemicals, Anaheim, Calif. 92805), ethylene glycol ("chromatoquality"; Matheson, Coleman and Bell, Norwood, Ohio 45212), and isooctane ("nanograde"; Mallinckrodt) were used without purification. Dichloromethane (Matheson, Coleman and Bell) was purified by passage through a column of silica gel (28–200 mesh, grade 12; Fisher Scientific Co., Pittsburgh, Pa. 15219).

Five-milliliter plastic disposable syringes fitted with 20-gauge needles (No. 805L/DN) were obtained from Becton-Dickinson Co., Rutherford, N. J. 07073. "Celite 545" was purchased from Fisher Scientific and bovine \(\gamma\)-globulin fraction II from Miles Laboratories, Elkhart, Ind. 46514. The polyethylene glycol reagent, used as a precipitant, was prepared as a 312 g/kg solution of polyethylene glycol ("Carbowax 6000"; Union Carbide Corp., San Francisco, Calif. 94138) in the buffer. Radioactivity was determined by using modified Bray’s solution (19), lacking methanol and ethylene glycol.

Celite liquid–liquid partition chromatography: Testosterone was separated from interfering steroids by our modified procedure for Celite column chromatography (20). The columns consisted of 5-ml plastic disposable syringes fitted with glasswool and packed first with 0.5 ml of Celite:water (2 g/ml) and then with 2 ml of Celite:ethylene glycol (2 g/ml).

Procedure

**Extraction and purification: 400–500 dpm (1.2–1.5 pg) of \([1,2-^3H]\text{testosterone}\), in 0.1 ml buffer was added to each of three scintillation vials and duplicate plasma samples (0.1 ml for men and 0.5 ml for women) and allowed to equilibrate for 1 h at room temperature. Then the samples were extracted with 5 ml of dichloromethane. The organic phase was dried under nitrogen, redissolved in 0.5 ml isooctane, and applied to prewashed Celite microcolumns (20). An additional 0.5 ml isooctane aliquot was used to transfer the remaining steroid. Interfering materials, notably dihydrotestosterone, were removed from the columns with 4 ml of isooctane followed by 5 ml of ethyl acetate in isooctane (50 ml/liter). Testosterone was then eluted from the columns with 6 ml of ethyl acetate in isooctane (150 ml/liter). This fraction was evaporated under nitrogen and redissolved in 1.0 ml of buffer. For use in estimating recoveries, 0.4 ml was transferred to a scintillation vial to which 10 ml Bray’s solution was added; 0.1- and 0.4-ml sample aliquots were used for assay purposes.

Assay procedure: The standard curve was prepared in triplicate by adding 0, 25, 50, 80, 120, 160, 200, 300, and 400 \(\mu\text{l}\) of the 1 ng/ml testosterone standard. After adding buffer to all samples to give a total volume of 0.4 ml, \([1,2-^3H]\text{testosterone}\) in 0.1 ml buffer was added to all tubes, which were shaken gently. Then 0.1 ml of diluted antiserum was added to all tubes except those used to calculate the total radioactivity added. Tubes were gently mixed and allowed to equilibrate at room temperature for 15 min and then at 0 °C for 2 h.

After the addition of 1 mg of bovine \(\gamma\)-globulin in 0.1 ml buffer and 0.5 ml of polyethylene glycol in buffer, the tubes were mixed on a vortex-type stirrer and centrifuged at 2800 rpm (1750 \(\times\) g) for 30 min at 0 °C. The supernate was decanted, 10 ml of Bray’s solution added, and the mixture was then counted for radioactivity.

Standard curves were constructed from the percent \[^3H\]testosterone antibody-bound, and plasma testosterone was calculated as follows: testosterone (\(\mu\text{g/liter}\)) = \(((0.1 \times X)/(P \times R \times B))\), where \(X = \text{pg}\) from standard curve, \(P = \text{volume of plasma assayed (ml)}, R = \%\) recovery, and \(B = \text{volume of buffer aliquot (ml)}\).

Results

Separation of Antibody-Bound and Free Hormone

Polyethylene glycol concentration: The solubility of testosterone in polyethylene glycol can be seen in Figure 1. Maximum precipitation of antibody-bound testosterone occurred with a final PEG concentration of 13–14 ml/dl with or without 200 pg of nonradioactive testosterone. In the absence of antibody, \[^3H\]testosterone was completely soluble in the presence (not shown) or absence of nonradioactive testosterone at 13–14% PEG. However, at higher PEG concentrations, a significant percentage of testosterone did not remain in the supernate. A final concentration of 13% PEG was selected.

Protein concentration: The precipitation of antibody-bound ligand with PEG depends on protein concentration. In the absence of all protein other than the antisera, there was no antibody precipitation. With gelatin in the buffer (1 g/liter) there was
marked, but sub-maximal, precipitation of antibody-bound testosterone in the absence of bovine γ-globulin (Figure 2). Maximum antibody precipitation occurred with approximately 0.75 mg of bovine γ-globulin per tube, with or without nonradioactive testosterone. Maximum precipitation persisted at higher concentrations of bovine γ-globulin. Free [3H]testosterone was completely soluble in the presence of 0 to 1 mg of bovine γ-globulin per tube, but slightly decreased solubility was observed with 1.5 mg. One milligram of bovine γ-globulin was chosen for all subsequent work.

Precipitation time: Two important time intervals were studied extensively to determine whether time was a critical factor in the PEG separation procedure. First, several standard curves were prepared as usual. After the PEG was added, each set of tubes was centrifuged after various time intervals. The initial percent bound (absence of nonradioactive steroid) did not change for the 4 h studied (Table 1); the corresponding standard curves were superimposable.

In a second experiment, the tubes containing the standard curves were allowed to remain at 0 °C, after centrifugation, for as long as 5.5 h. Again there was no apparent dissociation of antibody-bound testosterone, because the initial percent bound (Table 1) and standard curves were unchanged over this time period.

Assay Specificity

Antisera cross reactivity: The relative cross reactivity of various common plasma steroids with the antisera was determined by the method of Abraham (21). The specificity of the assay can be seen in Table 2. Dihydrotestosterone could replace [3H]testosterone nearly as well as testosterone, but other androgens and steroids demonstrated considerable less cross reactivity.

Potential sources of interference in plasma: If there were no purification procedures for removing interfering steroids before assay (assuming equivalent solvent extractability), dihydrotestosterone would increase normal plasma testosterone concentrations in men by 10–25% and 30–90% in women. Therefore, dihydrotestosterone must be removed before assay, if testosterone radioimmunoassay is to be accurate, especially for specimens from women.

The other common androgens would produce some interference in samples from men: androstenedione (1–2%), androstenediol (2–3%), and dehydroepiandrosterone (<1%). However, in women they would greatly increase the values for apparent plasma testosterone: androstenedione by 50%, androstenediol by 20–30%, and dehydroepiandrosterone by 5–10%. Therefore some form of separation, while highly desirable for plasma from men, is mandatory for an assay of testosterone in plasma from women.

Chromatography on Celite: To achieve specificity, we partially purified testosterone by solvent extraction followed by a modified Celite liquid–liquid partition chromatographic procedure (20). 60–70% of

| Table 1. Initial Percent of Testosterone Antibody Bound as a Function of Time in PEG Separation Procedure |
|-------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Time (h) | <0.1 | 0.25 | 0.5 | 1 | 3 | 3.5 | 4 | 5.5 |
| Delayed centrifugation | 71.9 | 71.8 | 72.5 | 70.9 | 70.0 | — | 70.4 | — |
| Delayed decantation | 71.3 | 70.9 | 70.1 | 71.3 | — | 72.8 | — | 71.0 |
Table 2. Specificity of Plasma Testosterone Radioimmunoassay

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross reactivity (percent)</th>
<th>Concentration (µg/liter)</th>
<th>Interference in plasma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No separation&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Testosterone</td>
<td>100</td>
<td>M 6.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 0.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>90.3</td>
<td>M 0.47–1.30</td>
<td>10–20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 0.16–0.34</td>
<td>30–90</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>12</td>
<td>M 0.75–1.05</td>
<td>1–2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 1.45–1.50</td>
<td>45</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>10.7</td>
<td>M 1.24–1.61</td>
<td>2–3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 0.68–0.96</td>
<td>20–30</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>0.8</td>
<td>M 1.82–5.32</td>
<td>0.2–0.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 2.87–4.80</td>
<td>5–10</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.02</td>
<td>M 0.21</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 0.47–7.65</td>
<td>0.0–0.43</td>
</tr>
<tr>
<td>17α-Hydroxyprogesterone</td>
<td>0.02</td>
<td>M 0.74</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F 0.40–15.6</td>
<td>0.02–0.8</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;&lt;0.01</td>
<td>M 150</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F</td>
<td>&lt;4</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Table 4.<br>
<sup>b</sup> On plasma, directly.<br>
<sup>c</sup> All values are maximal.<br>
<sup>d</sup> Negligible.

Plasma testosterone was routinely recovered after extraction and chromatography procedures. Less than 0.3% of the dihydrotestosterone, 0.1% of the androstenedione, 3% of the androstenediol, and 9% of the dehydroepiandrosterone were eluted in the testosterone fraction. Therefore, the combined interference for these steroids would normally be less than 10 ng per liter in specimens from persons of either sex. With the addition of the chromatographic step, the false increases in apparent plasma testosterone would be only 0.1–0.2% in men, as compared with 15–25% without chromatography, and 1–2% in women, as compared with >100% without chromatography.

Sensitivity, Blank, Precision, and Accuracy

Figure 3 shows a typical standard curve. The sensitivity of several standard curves, defined as the smallest amount that could be significantly discriminated from zero with 95% confidence, ranged from 3–20 pg; the average sensitivity was 9.1 pg. The actual assay aliquot contained about 25% of the original testosterone, because only 40% of the purified fraction was used in the assay (0.4 of 1.0 ml) and 60–70% was recovered. Therefore, the limit of detection for testosterone in a sample was 35 pg (350 ng per liter in men and 70 ng per liter in women).

When distilled water was treated similarly to plasma samples, the values varied about the sensitivity of the method. Occasionally these blank values rose to 20–40 pg, the cause invariably being the solvents; either use of fresh solvents, or repurification of the dichloromethane over silica gel decreased this source of error. Five-tenths milliliter of charcoal-treated plasma from women (28) was also assayed and the values averaged 11.4 pg for 19 samples.

The precision of the method was studied by repetitive measurement of testosterone in a pool of plasma from men, in replicate and from day-to-day. The intra- and interassay testosterone means for plasma from men were 4.49 ± 0.31 µg/liter (n = 8) and 4.79 ± 0.465 µg/liter (n = 15), respectively, with coefficients of variation of 6.9% and 9.7%, respectively. Corre-
Table 3. Recovery of Testosterone Added to Plasma

<table>
<thead>
<tr>
<th>Added (µl)</th>
<th>Expected (pg)</th>
<th>Observeda (pg)</th>
<th>Expected (pg)</th>
<th>Observeda (pg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63</td>
<td>58</td>
<td>23</td>
<td>13</td>
</tr>
<tr>
<td>50</td>
<td>113</td>
<td>108</td>
<td>73</td>
<td>76</td>
</tr>
<tr>
<td>100</td>
<td>163</td>
<td>190</td>
<td>123</td>
<td>130</td>
</tr>
<tr>
<td>150</td>
<td>—</td>
<td>—</td>
<td>173</td>
<td>170</td>
</tr>
<tr>
<td>200</td>
<td>263</td>
<td>273</td>
<td>223</td>
<td>247</td>
</tr>
<tr>
<td>250</td>
<td>—</td>
<td>—</td>
<td>273</td>
<td>266</td>
</tr>
<tr>
<td>300</td>
<td>—</td>
<td>—</td>
<td>323</td>
<td>332</td>
</tr>
</tbody>
</table>

* Expected pg obtained from mean of 13 determinations after charcoal treatment of samples.
* Mean of triplicate determinations.
* Mean of quadruplicate determinations.

Corresponding results for plasma from women were 0.531 ± 0.051 µg/liter (n = 6) and 0.63 ± 0.074 µg/liter (n = 13), with respective coefficients of variation of 9.6% and 11.8%.

The accuracy of the method was tested in two ways.

First, nonradioactive testosterone was added to plasma from men and women and the plasma was assayed (Table 3). The observed results were close to the expected values.

Second, increasing volumes of plasma from both men and women were assayed (Figure 4). Linear regression analysis calculated by the least-squares method resulted in a straight line with a correlation coefficient of 0.983 and y-intercept of 6.8 pg for plasma from men and 0.962 and -14 pg for plasma from women.

Normal Values

To further demonstrate the validity of the assay, determinations were performed on plasma from 24 healthy men between the ages of 21 and 65, and 20 healthy women of child-bearing age (Table 4). The mean values and ranges observed were comparable with values previously reported by other investigators using isotope derivative and radioimmunoassay procedures.

Discussion

Various methods for separating antibody-bound and free testosterone for radioimmunoassays have been reported, including methods that involve either nonspecifically adsorbing the free hormone with dextran-coated charcoal (11-17) or precipitating the antibody with either ammonium sulfate (10) or a second antibody (18). The dextran-coated charcoal technique has been most widely used.

In 1971, Desbuquois and Aurbach (32) demonstrated the use of PEG to separate antibody-bound and free hormone in polypeptide radioimmunoassays. In their procedure antibody-bound hormone was precipitated while the free hormone remained relatively soluble, depending upon the peptide studied and the conditions of the assay. Subsequently radioimmunoassays for other peptide hormones and low molecular weight substances (33, 34) have been developed in which PEG is used for antibody precipitation.

We recently reported the first use of PEG to separate antibody-bound and free ligand in a plasma steroid hormone radioimmunoassay (35). A specific, precise, and accurate assay for estradiol-17β was described. We have subsequently developed the reliable radioimmunoassay described here for plasma testos-

Table 4. Values for Plasma Testosterone Concentration

<table>
<thead>
<tr>
<th>Method of measurement</th>
<th>Mean (pg/ml)</th>
<th>± SD (pg/ml)</th>
<th>Range (pg/ml)</th>
<th>No.</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DID²</td>
<td>7.40</td>
<td>2.60</td>
<td>4.40-13.0</td>
<td>15</td>
<td>29</td>
</tr>
<tr>
<td>D1D</td>
<td>6.70</td>
<td>2.30</td>
<td>2.80-14.4</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>DID</td>
<td>5.75</td>
<td>—</td>
<td>—</td>
<td>23</td>
<td>31</td>
</tr>
<tr>
<td>RIA</td>
<td>5.90</td>
<td>1.49</td>
<td>3.65-8.15</td>
<td>13</td>
<td>32</td>
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<tr>
<td>RIA</td>
<td>7.90</td>
<td>1.43</td>
<td>4.50-11.0</td>
<td>30</td>
<td>32</td>
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<tr>
<td>RIA</td>
<td>4.90</td>
<td>1.60</td>
<td>2.50-8.80</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td>RIA</td>
<td>5.28</td>
<td>1.74</td>
<td>—</td>
<td>17</td>
<td>32</td>
</tr>
<tr>
<td>RIA</td>
<td>5.14</td>
<td>1.19</td>
<td>3.18-8.10</td>
<td>20</td>
<td>32</td>
</tr>
<tr>
<td>RIA</td>
<td>6.86</td>
<td>1.96</td>
<td>3.80-9.65</td>
<td>24</td>
<td>32</td>
</tr>
</tbody>
</table>

* DID, double isotope derivative technique; RIA, radioimmunoassay.
* Includes women on estrogen medication.
terone, with use of PEG to separate antibody-bound and free ligand, the second (to our knowledge) plasma steroid hormone radioimmunoassay to be described in which PEG is used.

Antibody precipitation was greatest with a minimum of 13–14% PEG for both steroid hormones. However, unlike estradiol-17β, free testosterone was precipitated to a small extent at higher PEG concentration. Nevertheless, the concentration of PEG necessary for optimum antibody precipitation was similar for testosterone and estradiol-17β. The difference between the apparent solubility of the two free hormones at high PEG concentrations may reflect their different polarities, testosterone being considerably less polar than is estradiol-17β. Radioimmunoassays of steroids more polar than estradiol-17β and less polar than testosterone are being developed to test this hypothesis.

We demonstrated that timing is not critical in this method of separation. There was no evidence of any dissociation of the antibody–hormone complex for several hours after PEG is added. Theoretically, the complex could have dissociated after precipitation and centrifugation of the antibody, but this too was not observed.

A chromatographic purification procedure is required to obtain a specific assay for testosterone because of the relative nonspecificity of the binding protein, whether it be sex steroid-binding plasma protein or an antibody. The former binds several plasma steroids in addition to testosterone, including dihydrotestosterone, estradiol-17β, and other 17β-ol steroids (36); it also has a greater binding energy for dihydrotestosterone than for testosterone (37).

Most testosterone antibodies have been produced by using testosterone-3-(O-carboxymethyl)oxime-bovine serum albumin conjugates as immunogen. The most significant interfering steroid from plasma has generally been 5α-dihydrotestosterone, a finding confirmed with the antibodies we tested. For this reason, steroid separations are primarily directed to resolve dihydrotestosterone and other 17β-ol androgens from testosterone before the sample is incubated with the antisera. This separation has been achieved by thin-layer chromatography (11) or column chromatography on alumina (10), Sephadex LH-20 (14), or Celite (12).

The latter two methods, with use of glass columns, have been the most popular. We achieved specificity with a modified Celite micro-column procedure in which plastic syringes were used as columns. These columns are easier to fill and require no external pressure, yet the flow rates are relatively rapid. No significant blank was produced from this procedure, and more than 90% of the testosterone could be analytically accounted for in the eluate.

Attempts have been made to obtain antibodies of higher specificity by preparing antigens conjugated to testosterone in areas other than carbon 3. Theoretically this could permit the development of specific testosterone radioimmunooassays without a chromatographic step. However, antigens conjugated to testosterone at C 11 or C 17 (38), C 6 (39), or C 7 (40) have produced antibodies that still retain a high cross reactivity to dihydrotestosterone. Thus for the near future it appears that some form of chromatography will be required to achieve high specificity routinely.

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References