NON-POLAR EXTRACTS OF SERUM FROM MALES CONTAIN COVERT RADIOIMMUNOASSAYABLE TESTOSTERONE

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ABSTRACT

Non-polar extracts of sera from human males contain immunoreactive testosterone in a form that is released by mild alkaline hydrolysis. The non-polar derivative shows no immunoreactivity with testosterone antibody prior to hydrolysis. Hydrolyzed non-polar serum extracts from ten adult male volunteers contained 2.0 ± 0.8 (SD) ng/mL of testosterone. Neither non-polar serum extracts of normal females nor a water blank substituted for non-polar extract of serum yields any immunoreactive testosterone after alkaline hydrolysis. Testosterone palmitate hydrolyzed alone or after addition to non-polar extract of serum yields the expected quantities of radioimmunoassayable testosterone. Previously described conjugates of testosterone are polar and are neither extractable by petroleum ether nor hydrolyzable by alkali. These observations suggest that fatty acid esters of testosterone may be present in serum of human males.

INTRODUCTION

Non-polar esters of estradiol have been isolated and

characterized from several tissues including human breast tumors (1-3). These esters have been found to be long-acting estrogens (4). Acyl CoA/acyl-transferases that catalyze fatty acid conjugation are present in microsomal fractions of human mammary cancer cells (5). Intracellular carboxy esterases are also known to occur in MCF-7 cells, a human breast tumor cell line (6). These findings support the view that non-polar steroid esters act as reservoirs, and are hydrolyzable by endogenous esterases, to yield the free steroid (2). In vitro experiments have also shown that the non-polar estradiol fatty-acid esters are hydrolyzable by alkali to estradiol (2). It has recently been demonstrated that free fatty acids such as linoleic and palmitic, at physiologic concentrations, inhibit the binding of testosterone to albumin and sex hormone-binding globulin (SHBG). This suggests that fatty acids may be important regulators of bioavailable testosterone (7). In the present study, an adaptation of the method of Hochberg et al (1) was used to demonstrate that mild alkaline hydrolysis releases free testosterone from acetate, propionate, palmitate and enanthate esters, non-polar extracts of human male serum, but not from non-polar extracts of normal human female serum.

MATERIALS AND METHODS

Testosterone, testosterone propionate, and testosterone enanthate were purchased from Sigma Chemical Co. (St. Louis, MO). [4-^{14}C]testosterone (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Sera were obtained from human males at least 20 years old and from normal females, 25 years or older. Testosterone palmitate was prepared by refluxing 10 mg testosterone with an excess of palmitic anhydride (30 mg) in 1 mL of freshly distilled pyridine for 3 h. Twenty milliliters of 10% hydrochloric acid was added and the mixture then extracted with petroleum ether, which was washed with 10% sodium bicarbonate and distilled water. The petroleum ether was then washed twice with 50% aqueous methanol to remove any unreacted testosterone, evaporated, and the residue purified by high-pressure liquid chromatography.

[4-^{14}C]testosterone acetate was prepared by refluxing acetic anhydride with carrier testosterone and [4-^{14}C]testosterone as described for testosterone palmitate. The esters had: Rf values less polar than testosterone on TLC, absorption peaks at 240 nm; they did not react with anti-testosterone antibody and gave an alkaline hydrolytic product with Rf identical to testosterone. Acetonitrile (HPLC grade) and methanol were purchased from Fisher Scientific Co. (Pittsburg, PA). The μBondapak CN Reverse Phase Column (7.8 mm x 30 cm) and Acro LC35 Filters (0.2 μm) were purchased from Waters Associates (Milford, MA). Testosterone Radioimmunoassay (RIA) Kits were obtained from Wien Laboratories (Succasunna, NJ).

Chromatography

Reverse phase high-pressure liquid chromatography (RP-HPLC) was used to purify all hydrolyzed samples before RIA. Samples were isocratically eluted from a μBondapak CN column with acetonitrile/water (1:1) at 2 mL/min [System I]. Equal volumes of the pre-testosterone, testosterone, and post-testosterone fractions (A, B, and C, respectively) were assayed for testosterone by RIA. The isocratic systems were selected by adjusting solvent ratios to give wide separation of free and esterified testosterone at reasonable retention times. Solvent system I (methanol/acetonitrile/water) [2:1:1] was used to purify synthesized testosterone palmitate. Absorbance was monitored at 240 nm.

Extraction and hydrolysis

Ten milliliters of serum samples were extracted with 20 mL petroleum ether (petroleum ether was selected because extraction with two volumes removed only 7% of free testosterone from serum). The petroleum ether was evaporated and the residue taken up in 2 mL methanol. To aliquots of the sample to be hydrolyzed was added an equal volume of 2% methanolic potassium hydroxide (KOH) or for a mock hydrolysis control, an equal volume of methanol. Incubation, at 45 C, was done in an atmosphere of nitrogen for 3 h, after which 10 mL of water were added and the mixture extracted with 3 mL methylene chloride. The methylene chloride was evaporated and the residue taken up in 0.5 mL acetonitrile/water (1:1) for HPLC purification as described above. Alkaline hydrolysis was done under nitrogen to minimize destruction of testosterone. The time and temperature used were those that completely hydrolyzed the model testosterone esters.

Testosterone palmitate (T-pal) recovery from non-polar serum extract

Two hundred microliters of non-polar serum extract (equivalent to 0.8 mL serum) were hydrolyzed or mock hydrolyzed. Exactly 3.9 ng of spectrophotometrically quantitated T-pal were similarly hydrolyzed or mock hydrolyzed. Another 200 μL of the same non-polar serum extract, to which 3.9 ng T-pal had been added, was also hydrolyzed or mock hydrolyzed. The hydrolysates were then extracted with 3.0 mL of methylene chloride. Exactly 2.4 mL of the methylene chloride was evaporated and the residue taken up in 0.5 mL acetonitrile/ water (1:1) of which 0.4 mL was loaded on the HPLC column for purification. Duplicate aliquots (400 μL, 200 μL) of the fraction in which testosterone elutes (B) and adjacent fractions (A and C) were assayed for testosterone by RIA, at two levels.

Radioimmunoassay

The method described by Wien Laboratories was used to assay 500 μL and 250 μL aliquots of HPLC fractions A, B, and C in duplicate. The evaporated aliquots of the appropriate fractions were dissolved in phosphate saline buffer containing 0.06% BSA

and incubated with antibody and radioligand for 1 h (4 C). Coated charcoal was used to separate bound and free steroid. The antibody is highly specific for testosterone and only moderately reacts with dihydrotestosterone (DHT). It does not cross-react with testosterone esters, even at 2 ng per assay tube level. RIA data from standards, controls, and samples were analyzed by a Beckman LS3801 liquid scintillation counter equipped with a data reduction system for linear regression plot of [Bound/max Bound] on dose (pg/tube). The program used in the construction of the standard curve rejects data points outside 95% confidence limits. Control samples (0, 200, and 400 pg testosterone) were assayed with each run. The inter-assay and intra-assay %CV were 12.7% (n=6) and 7.0% (n=6), respectively.

RIA of authentic testosterone added to fraction B

To assess if the observed increase in radioimmunoassayable testosterone was due to an artifact, known amounts of authentic testosterone (200 pg) were added to fraction B of hydrolyzed non-polar serum extracts, then radioimmunassayed as described above.

RESULTS

Preliminary experiments clearly demonstrated that the antibody did not cross-react with testosterone fatty acid esters. The hydrolysis or mock hydrolysis of water showed no detectable testosterone in any of the fractions. The alkaline hydrolytic treatment of 3.0 ng of authentic testosterone, purification by HPLC, and assay by RIA, however, showed immunoassayable testosterone only in fraction B, but not in fractions A or C. Recoveries of 90% of testosterone for "mock hydrolyzed" and 75% for "hydrolyzed" samples demonstrated that "hydrolysis"

did not cause artifactual increases in the amounts of testosterone detected by the antibody (Table 1).

Table 1. THE EFFECT OF "HYDROLYSIS" ON AUTHENTIC TESTOSTERONE

Sample	Observed (pg/tube)	Expected (pg/tube)	% Recovery
Mock hydrolyzed	180 ± 7.0	200	90
Hydrolyzed	150 ± 4.2	200	75
Distilled water	0*	0	

Known amounts of authentic testosterone were "hydrolyzed" or "mock hydrolyzed," purified, and then assayed by RIA. Testosterone was detected in fraction B only. The values given are the mean ± SD. *=below level of detection.

In order to show that hydrolysis of serum extracts or subsequent purification did not produce some substance which led to overestimation of radioimmunoassayable testosterone, known amounts of testosterone were added to fraction B of hydrolyzed non-polar extracts. An average of 95% of the added testosterone was recovered. HPLC elution times for testosterone, testosterone acetate, testosterone propionate, and testosterone enanthate were 8, 11.5, 12.5, and 17.2 min, respectively (Figure 1). Mock hydrolyzed [4-^{14}C]testosterone acetate eluted at the same time as the unhydrolyzed ester (11 min). The elution time after hydrolysis corresponded to the time for free

testosterone. Recoveries, in terms of radioactivity, were 79% and 96% for hydrolyzed and mock hydrolyzed samples, respectively. No testosterone was found in mock hydrolyzed non-polar serum extracts, testosterone palmitate samples, sera to which testosterone palmitate was added, distilled water or hydrolyzed non-polar extracts from normal human females. Following hydrolysis, testosterone was recovered from non-polar serum extracts, testosterone palmitate samples, and non-polar serum extracts with added testosterone palmitate (Table 2).

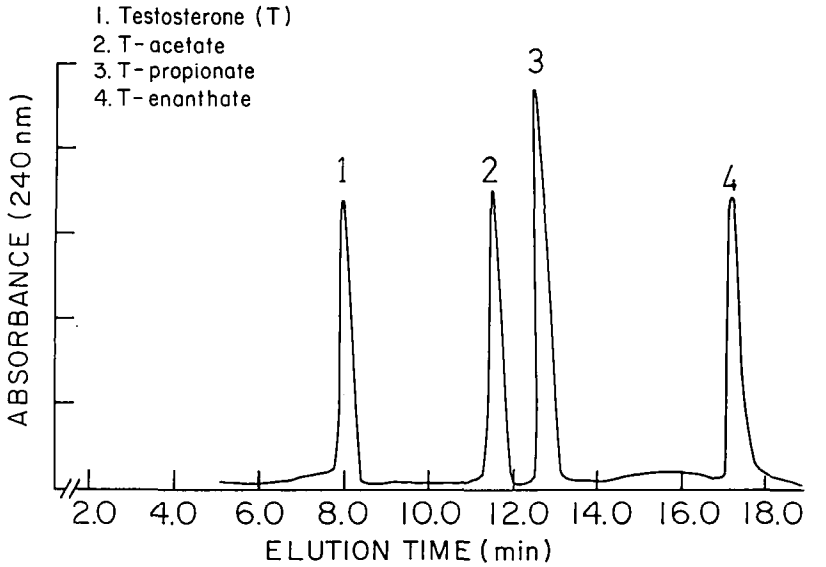

Figure 1. RP-HPLC of testosterone and some testosterone esters. Samples were isocratically eluted from a μBondapak CN column with acetonitrile/water (1:1) at 2 mL/min. A full scale deflection of 10 represents an OD of 1.0.

Table 2 RECOVERY OF ADDED T-PAL FROM NON-POLAR EXTRACT

	Found (pg/tube)	Expected (pg/tube)	% Recovery
NP extract	70 ± 3.0	-	-
T-pal	284 ± 12	248	114
	159 ± 6	124	128
NP + T-pal	349 ± 16	318	109
	183 ± 11	159	115

Known amounts of T-pal were hydrolyzed with or without addition of a non-polar extract. Fractions A and C contained no detectable testosterone nor did reaction mixtures incubated omitting KOH. The testosterone content of fraction B is shown as mean ± SD. NP= non-polar serum extract.

Testosterone was found only in fraction B of all hydrolyzed non-polar extracts of male serum but not in mock hydrolyzed samples (Table 3).

Table 3. RADIOIMMUNOASSAYABLE TESTOSTERONE IN NON-POLAR EXTRACTS OF SERA FROM HUMAN MALES AND FEMALES

Sex	Immunoassayable T
Male	2.0 ± 0.8 ng/mL
Female	0.1 ± 0.3 ng/mL

Radioimmunoassayable testosterone in hydrolyzed non-polar serum extracts. Eight of ten female samples showed no detectable testosterone in hydrolyzed non-polar extracts. The values given are the mean ± SD. T= testosterone.

DISCUSSION

Non-polar conjugates of estradiol have been well characterized (1-3) and shown not to bind directly to estrogen receptors (9). Studies in rats indicate that C-17 estradiol esters exert long range estrogenic effects (4) because of slow hydrolysis in vivo (8). It is known, however, that as the fatty acid moiety increases beyond C-12, there is a rapid increase in the clearance of estrogen esters from blood (8). These non-polar estradiol esters are known to be hydrolyzable by alkali, to yield the free steroid (2).

When sera from male subjects were extracted with petroleum ether, a non-polar form of testosterone (NPT) was obtained. Prior to hydrolysis, the non-polar substance described herein did not react with an antibody that is specific for testosterone. However, when the petroleum ether extract was hydrolyzed as described, radioimmunassayable testosterone was obtained. The hydrolytic product reacted with specific antibody for testosterone, eluted in the appropriate fraction on RP-HPLC, and has been tentatively identified as testosterone, pending GC-mass spectrometer analysis. We investigated the possibility that the apparent immunoassayable steroid was an artifact. We showed

that when testosterone itself was "hydrolyzed," purified by HPLC, and assayed by RIA, artificially high amounts of testosterone were not detected. The HPLC system clearly spearated testosterone from its esters. Thus when equal volumes of pre-testosterone, testosterone, and post-testosterone fractions (A, B, C, respectively) were assayed by RIA, only the testosterone peak gave any detectable immunoassayable steroid. Some freshly distilled water was hydrolyzed or mock hydrolyzed as described in the Materials and Methods section. None of the HPLC fractions showed any detectable testosterone.

T-pal was used as a model compound and was recovered as testosterone in expected amounts only in fraction B of hydrolyzed samples. T-pal, being recoverable in expected quantities and in fraction B, underscored the assertion that the hydrolysis step allowed satisfactory detection.

Radioimmunoassay of known amounts of testosterone added to fraction B from hydrolyzed serum extracts also yielded recoveries of about 95%. This showed that the detected steroid is not due to "injury" of the antibody from some unknown product of hydrolysis.

It has recently been shown that free fatty acids at physiological levels may play a role in the bioavailablity of

testosterone to tissues (7). It was asserted that unsaturated free fatty acids can inhibit the binding of testosterone to albumin and sex hormone binding globulin (SHBG). Acyl CoA/acyl-transferases may thus be able to catalyze the conjugation of free fatty acids to testosterone at the C-17 position. It has been suggested that fatty Acyl CoA/estradiol-17ß acyl-transferase in bovine placenta can use testosterone and androstanediol as substrates (13).

Long-chain fatty acid conjugates of ecdysone have been isolated from invertebrates such as *Boophilus microplus* (10), adult female cricket, *Gryllus bimaculatus* (11), the house cricket, *Acheta domesticus* (12), and possibly different classes of arthropods. The endogenous conjugation of long-chain fatty acids to steroids may be more widespread than hitherto observed.

We propose the existence of non-polar form(s) of testosterone (NPT) in serum from human male. Since internal standards are not used, the amounts of NPT we have estimated may be less than is actually present in blood. Levels would be better estimated when the exact nature of NPT has been

determined by mass spectrometry. The levels of NPT determined (2.0 ± 0.8 ng/mL) are in the lower range of plasma testosterone levels (3-10 ng/mL) in adult human males. However, they far exceed castrate levels (<0.5 ng/mL) achieved in androgen suppression therapy of prostatic cancer patients. In a report to be published separately, we will show that NPT occurs even in such patients. The testosterone conjugate described herein did not react with specific antibody for testosterone prior to hydrolysis. In common with estradiol esters, they may serve as reservoir forms of androgen that exert long-range effects.

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