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Serum and Urinary Markers of Exogenous Testosterone Administration

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In an attempt to find optimal markers of exogenous testosterone (T) administration in male athletes, a number of compounds were measured in 11 healthy men before and after 3, 6 and 9 months of weekly administration of 250 mg i.m. T enanthate and in age-matched untreated controls. The following variables were measured in serum: T, 17α-hydroxyprogesterone (17-OHP), sex hormone-binding globulin (SHBG), estradiol-17β, estrone (free + conjugated) and luteinizing hormone (LH). The following variables were measured in urine: T glucuronide (urinary T), epitestosterone glucuronide (urinary epiT), estrone (free + conjugated) and LH. Serum T, serum T/17-OHP ratio, serum T/LH ratio, serum T/SHBG ratio, serum and urinary estrogens , urinary T/creatinine-, T/epiT- and T/LH ratios increased whereas serum 17-OHP, SHBG and LH and urinary epiT/creatinine- and LH/creatinine-ratios decreased significantly during treatment. Levels above the upper reference limit were found in all subjects at 3, 6 and 9 months for serum T/17-OHP and serum and urinary T/LH ratios and at 6 months for the urinary T/epiT ratio. Levels below the lower reference limit were found in all subjects at 3, 6 and 9 months for serum LH and the urinary LH/creatinine ratio, at 3 months for the urinary epiT/creatinine ratio and at 9 months for serum 17-OHP. No other variable showed abnormal values in all subjects at the same occasion. Despite significant changes during treatment, steroid concentrations as such are poor indicators of T doping. Serum and urinary LH levels, T/LH ratios and serum T/17-OHP ratios seem to be the most reliable markers of exogenous T administration in males.

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INTRODUCTION

Epitestosterone (epiT, 17α-hydroxy-4-androstene-3-one), an epimer of testosterone (T), is mainly formed in the testis and is secreted in its free form and as its sulfate [1]. Exogenous administration of T or other androgenic–anabolic steroids distinctly suppresses testicular secretion of epiT and very little, if any, circulating T is converted peripherally into epiT. Exogenous T administration thus leads to a markedly increased ratio between circulating T and epiT. An elevated ratio between the glucuronides of T and epiT in urine (T/epiT ratio) has been accepted as a marker for T doping by the International Olympic Committee (International Olympic Committee Medical Committee Meeting in Los Angeles, CA, February 1982). However, in certain rare cases epiT production and metabolism may be affected by genetic, environmental or dietary factors, leading to extremely low epiT values and false-positive tests [2–5]. This prompted us to search for other markers of T doping in both serum and urine.

17α-Hydroxyprogesterone (17-OHP) is an intermediate in testicular androgen synthesis, and testicular secretion accounts for about 75% of circulating 17-OHP in healthy men [6]. Administration of synthetic androgenic–anabolic steroids has been shown to suppress serum levels of 17-OHP [7,8]. In a recent communication [9], we reported the ratio between circulating levels of T and 17-OHP to be a good marker of T doping after a single injection of 250 mg T enanthate. The validity of the serum T/17-OHP ratio as a marker of T doping was further supported by the finding of perfectly normal values in a subject with a well documented, constantly elevated urinary T/epiT ratio not related to exogenous T administration (for

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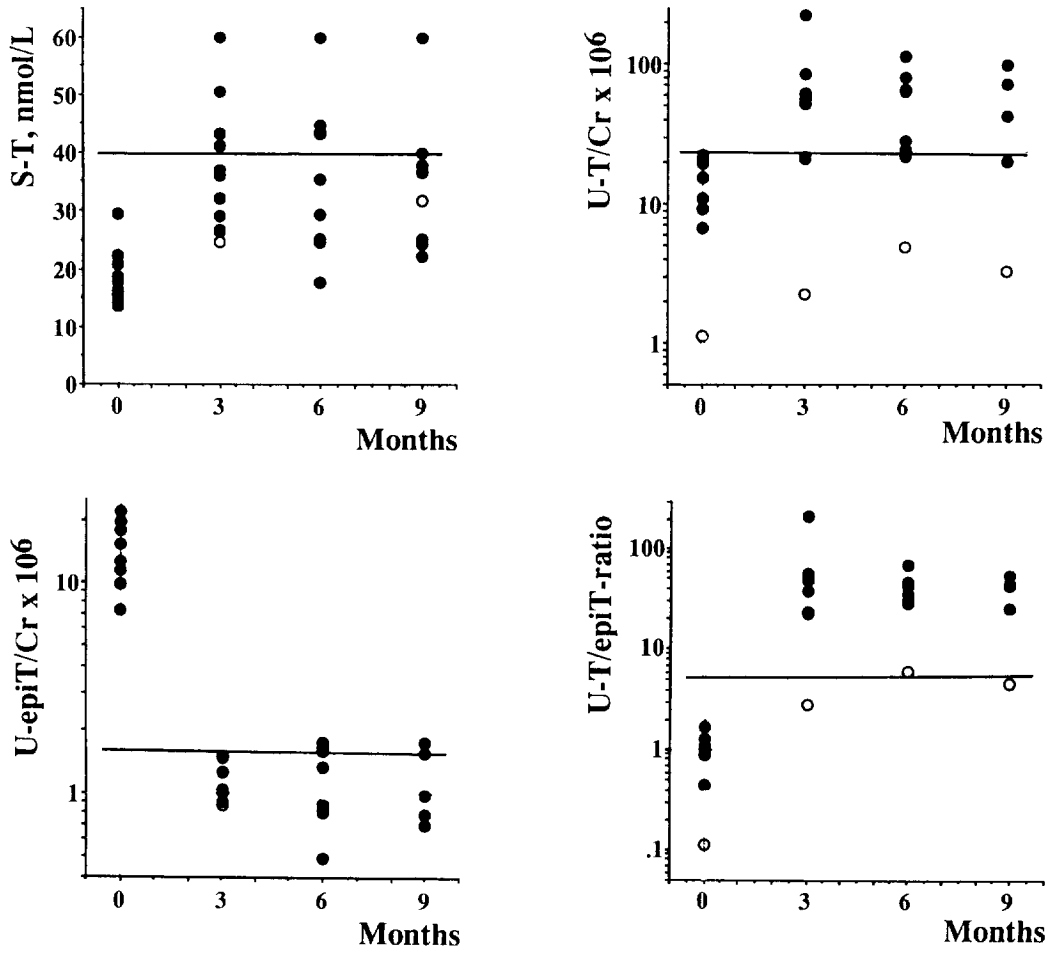

Fig. 1. Serum concentrations of T (S-T) and urinary T glucuronide/creatinine (U-T/Cr) and epiT glucuronide/creatinine ratios (U-epiT/Cr) and urinary T/epiT ratio (U-T/epiT) in healthy men before and during 9 months of weekly injections with 200 mg of T enanthate. The horizontal line indicates the upper reference level for S-T, U-T/Cr and U-epiT/T and the lower reference level for U-epiT/Cr. The open circles indicate a subject with consistently low U-T/epiT ratios.

details of this case, see [4]). In order to validate the serum T/17-OHP-ratio as a marker of T doping also in a long-term study, we have followed the serum and urinary hormone patterns in healthy male volunteers during 9 months of weekly injections of 200 mg of T enanthate.

MATERIALS AND METHODS

Subjects

The study population comprised 11 healthy sedentary men participating in a WHO investigation program for male contraception. They received an i.m. injection of 200 mg T enanthate (Testoviron-Depot®, Schering AG, Berlin, Germany) every week for 9 months. Samples of venous blood and urine (untimed samples) were collected 1 week before and after 3, 6 and 9 months of treatment. The blood samples were collected at 0800 a.m. Serum and urine were stored at −20°C and all samples from the same subject were analyzed in the same assay in order to avoid between-assay variation.

Reference values

Normal ranges for most endocrine variables were obtained from an age-matched population of healthy sedentary men, selected according to the same inclusion criteria as above. The reference population has been decribed in detail [9]. In some cases the reference material was extended, giving slightly changed reference limits. The "reference limits" given in Figs 1–4 refer to the highest or lowest value observed in the reference population.

Analytical methods

Serum T, 17-OHP, estradiol-17β (E_2), serum and urinary total estrone (tE_1; sum of free and conjugated estrone, 85% estrone sulfate) and serum and urinary luteinizing hormone (LH) were determined by radioimmunoassay or (LH) fluoroimmunoassay methods [9]. Serum SHBG was determined immunoradio-

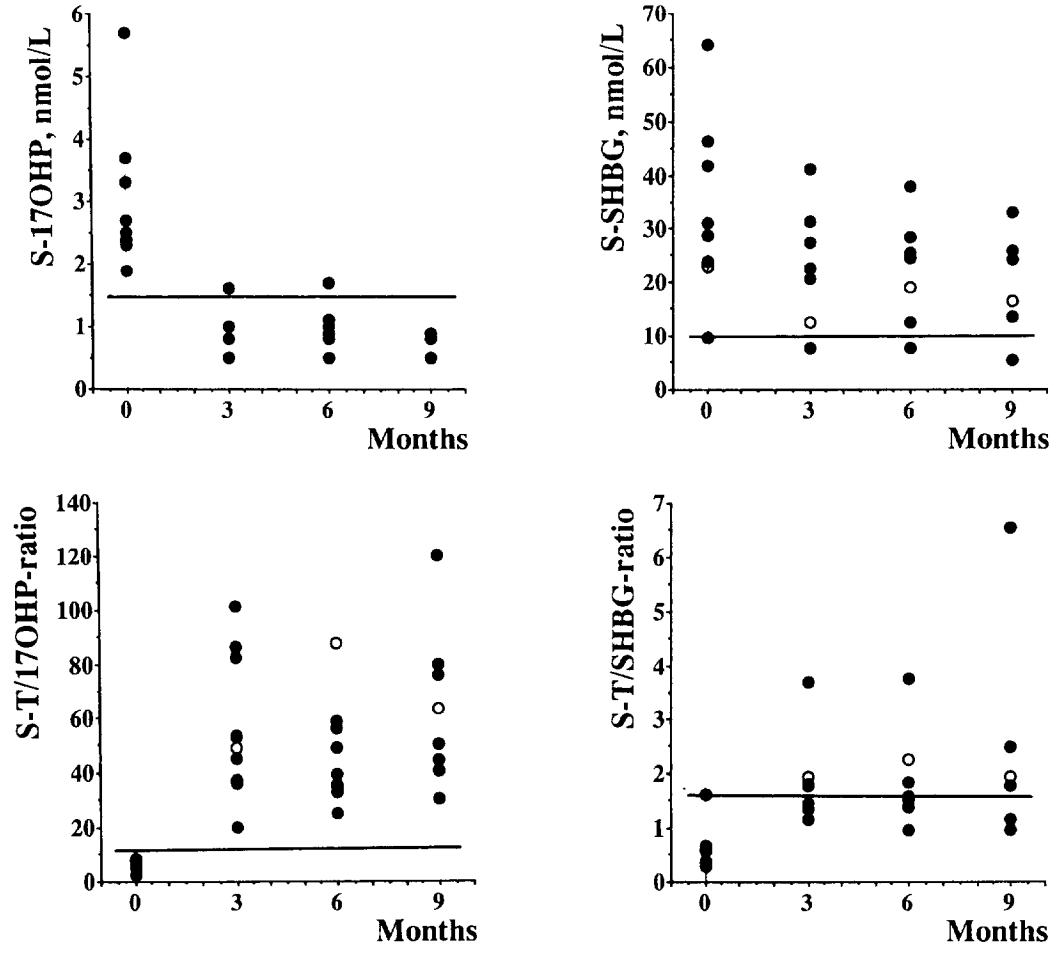

Fig. 2. Serum concentrations of 17α-hydroxyprogesterone (S-17-OHP), sex hormone-binding globulin (S-SHBG), serum T/17-OHP and T/SHBG ratios in healthy men before and during 9 months of weekly injections with 200 mg of T enanthate. The horizontal lines indicate the upper reference level for S-T/17-OHP and S-T/SHBG ratios and the lower reference level for S-17-OHP and S-SHBG. The open circles indicate a subject with consistently low U-T/epiT ratios.

metrically using a commercial kit obtained from Orion Diagnostica Oy, Turku, Finland. Urinary T glucuronide (urinary T) and epiT glucuronide (urinary epiT) were determined after hydrolysis by GLC–MS according to Donike and co-workers [10] with minor modifications. Data on minimum quantifiable concentrations as well as within- and between-assay coefficients of variation have been given previously for all methods except SHBG [10]. For SHBG the minimum quantifiable concentration was set at 6.3 nmol/l (lowest point in the calibration curve) and within- and between-assay coefficients of variation were 3 and 6%, respectively.

Because untimed urinary samples were used, urinary T, epiT, tE_1 and LH concentrations were related to urinary creatinine content (Hormone/Cr ratios). The ratio between serum T and SHBG was used as an index on biologically active T. This index has been shown to correlate well to free or non-SHBG-bound T determined by physicochemical methods [11, 12].

Statistical methods

Changes in endocrine variables during treatment were tested for significance by analysis of variance (ANOVA). Correlations were tested by Spearmans rank correlation test. Normally distributed values are given as arithmetic mean and SEM, otherwise as median and range.

RESULTS

Levels of serum and urinary hormones as well as hormone ratios during the observation period are shown in Table 1 and Figs 1–4. Serum levels of T, E_2 and tE_1 as well as urinary T/Cr and tE_1/Cr were significantly increased whereas serum levels of 17-OHP, SHBG and LH, as well as urinary epiT/Cr and LH/Cr, were significantly decreased after 3, 6 and 9 months. Serum T/17-OHP, T/SHBG and T/LH, as well as urinary T/epiT and T/LH, ratios were significantly increased during the entire period of treatment.

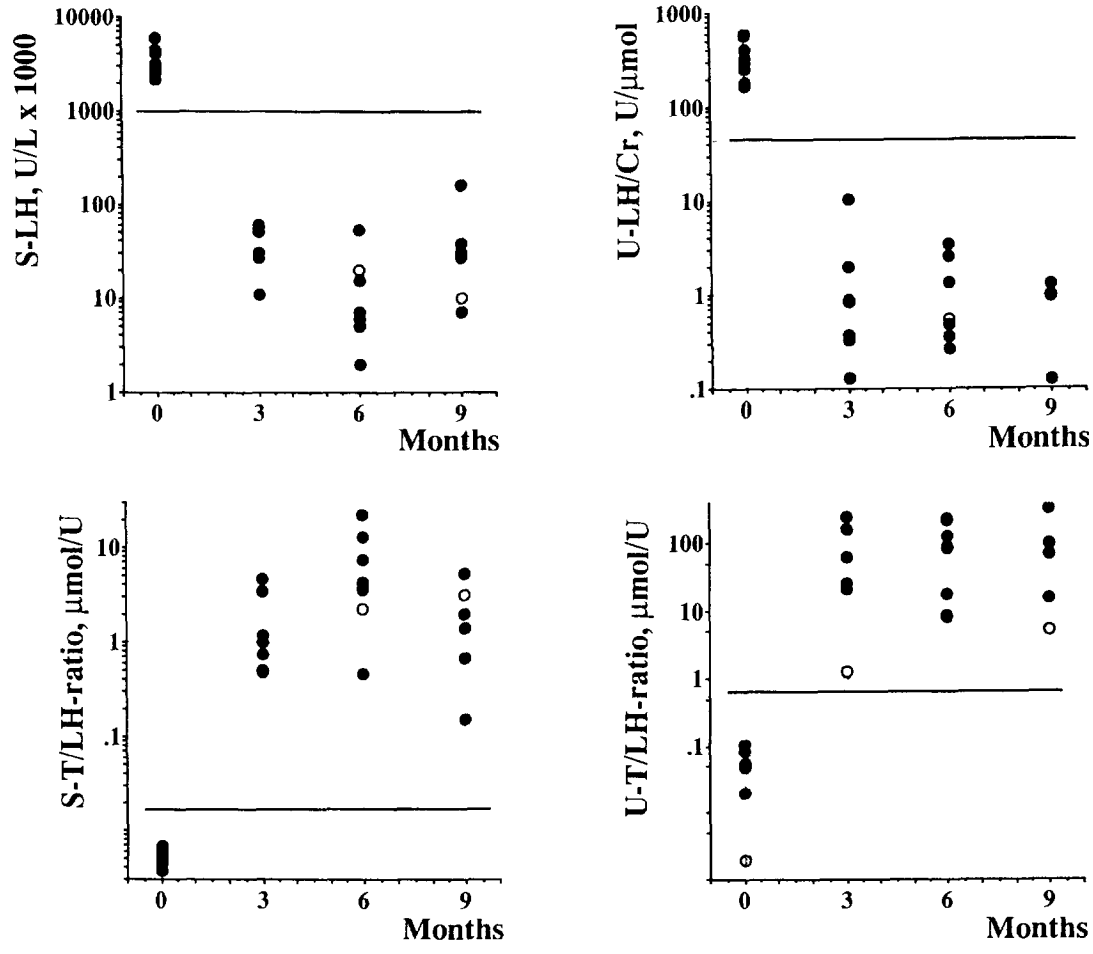

Fig. 3. Serum concentrations of LH (S-LH), urinary LH/creatinine (U-LH/Cr) and serum and urinary T/LH ratios in healthy men before and during 9 months of weekly injections with 200 mg of T enanthate. The horizontal line indicates the lower reference level for S-LH and U-LH/Cr and the upper reference level for serum and urinary T/LH ratios. The open circles indicate the subject with consistently low U-T/epiT ratios.

The number of subjects showing abnormal values during T enanthate administration are given in Table 2. Serum LH and urinary LH/Cr values were decreased whereas serum T/17-OHP and serum and urinary T/LH-ratios were elevated in all subjects during the entire period studied. No other variable, including the urinary T/epiT ratio, showed abnormal values in all subjects at all three occasions. From 3 months and onwards, serum 17-OHP was suppressed to a mean level corresponding to 23±2%, urinary

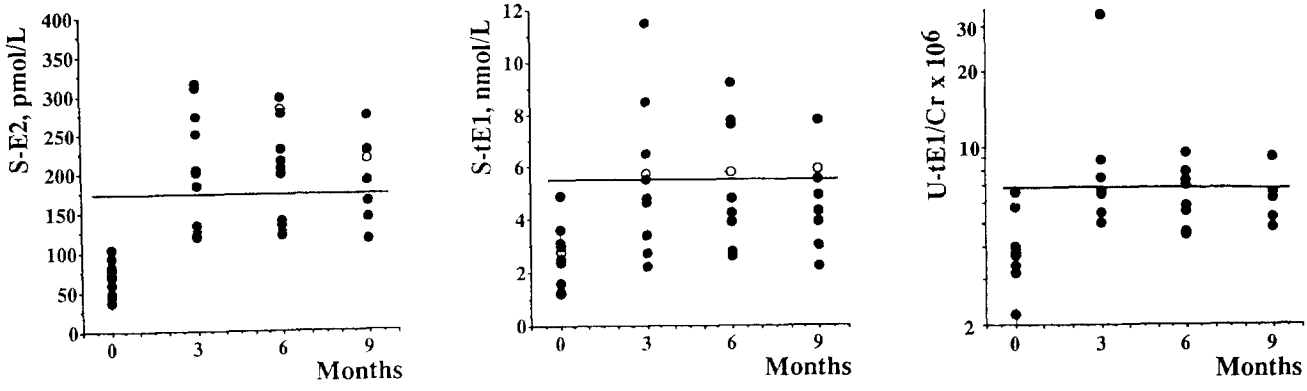

Fig. 4. Serum concentrations of E_2 (S-E2), total estrone (S-tE_1) and urinary tE_1/creatinine ratio (U-tE_1/Cr) in healthy men before and during 9 months of weekly injections with 200 mg of T enanthate. The horizontal lines indicate upper reference levels.

Table 1. Serum and urinary steroids and LH and endocrine ratios in healthy men before and during 9 months of weekly treatment with 200 mg of i.m. enanthate

	0 months	3 months	6 months	9 months
S-T (nmol)	17.9 ± 1.5	37.0 ± 3.3**	35.4 ± 4.0**	34.8 ± 4.3**
S-17OHP (nmol/l)	3.1 ± 0.4	0.8 ± 0.1***	0.8 ± 0.1***	0.6 ± 0.1***
S-LH (U/l × 1000)	1950 (2200–6000)	40 (11–60)***	6 (2–53)***	29 (7–158)***
U-LH/Cr (U/μmol)	310 (170–595)	1.0 (0.2–10.0)**	1.0 (0.1–3.0)**	1.0 (0.1–1.0)**
S-E_2 (pmol/l)	68 ± 6	210 ± 21***	210 ± 21***	189 ± 20***
S-tE_1 (nmol/l)	2.7 ± 0.3	5.5 ± 0.8**	5.8 ± 0.8***	4.7 ± 0.6**
U-tE_1/Cr	3.82 (2.20–6.58)	6.61 (4.99–6.61)***	6.46 (4.50–9.45)**	6.26 (4.77–9.00)***
S-SHBG (nmol/l)	33.7 ± 5.9	23.5 ± 4.3*	22.3 ± 3.8**	19.8 ± 4.0**
S-T/SHBG ratio	0.58 (0.29–1.62)	1.77 (1.18–3.70)*	1.59 (0.96–3.75)*	1.87 (0.88–6.54)**
U-T/Cr	13.1 (1.1–22.0)	53.8 (2.5–223)***	45.3 (4.8–112)***	42.0 (3.2–96.2)***
U-epiT/Cr	13.8 (7.4–21.9)	1.0 (0.9–1.5)***	1.1 (0.5–1.8)***	1.0 (0.7–1.8)***
S-T/17-OHP ratio	6.3 (2.8–8.3)	49.2 (20.2–101)***	38.0 (25.2–87.4)**	57.3 (30.4–121)***
U-T/epiT ratio	0.94 (0.13–1.68)	43.2 (2.9–214)**	39.8 (6.0–70.2)**	42.6 (4.6–54.9)**
S-T/LH ratio (μmol/U)	0.005 (0.004–0.007)	0.98 (0.48–4.60)***†	4.11 (0.47–22.45)***†	1.70 (0.15–5.23)***†
U-T/LH ratio (μmol/U)	0.052 (0.002–0.108)	45.16 (1.28–252)***†	85.7 (8.3–238)***†	71.7 (5.3–344)***†

The values are given as mean and SEM or median and range according to distribution. Significant group dierences from pretreatment values are denoted by *P>0.05; **P>0.01 and ***P>0.001, respectively (ANOVA). †After logarithmic transformation.

epiT/Cr to 9 ± 1%, serum LH to 0.11 ± 0.03% and urinary LH/Cr to 0.04 ± 0.01% of pretreatment values.

DISCUSSION

The results of the present study support the general concept presented by Donike [13], that the ratio between the level of T and that of some other compound involved in the pituitary–testicular activity, may be used as a marker of T doping. More specifically, our findings evidently confirm the results from our previous short-term study, with a less heavy exposure to T [9], showing the T/17-OHP ratio in serum to be an excellent marker of T doping. In contrast to the short term study, also serum and urinary LH were dramatically decreased and the T/LH ratios were elevated in all subjects during long term T administration. This further supports the value of LH determinations and the T/LH ratio for detection of T doping [14].

Table 2. Number of subjects having abnormal† serum and urinary hormone levels during 9 months of weekly treatment with 200 mg of i.v. enanthate

	3 months	6 months	9 months
S-T	4/11	4/10	1/8
S-17-OHP	9/11	10/11	8/8*
S-T/17-OHP	11/11*	10/10*	8/8*
S-SHBG	1/8	1/7	1/7
S-T/SHBG	5/8	3/8	4/7
S-LH	8/8*	8/8*	6/6*
S-T/LH	8/8*	8/8*	6/6*
S-E_2	7/11	7/10	4/8
S-tE_1	4/11	5/10	2/8
U-T/epiT	7/8	8/8*	4/5
U-LH/Cr	8/8*	8/8*	5/5*
U-T/LH	8/8*	8/8*	5/5*
U-tE_1/CR	1/8	4/8	1/5

*Abnormal values in all subjects.

†Above the "normal range" (highest individual value in the reference population) except for S-17-OHP, S-SHBG, S-LH and U-LH/Cr.

Of the different ratios used or proposed as markers of exogenous T administration, the urinary T/LH ratio showed the most dramatic increase in the present study—about 1000-fold—followed closely by the serum T/LH ratio. The urinary T/epiT and the serum T/17-OHP ratios increased more modestly, about 50- and 10-fold, respectively. The relatively modest increase in the serum T/17-OHP ratio reflects the adrenocortical contribution to circulating levels of this steroid, which is certainly not affected by T administration. The levels of circulating 17-OHP remaining after T enanthate administration are close to those found after orchidectomy [15], indicating an almost total suppression of testicular steroid secretion.

Concerning urinary epiT, the situation is more complex. Urinary epiT/Cr decreased to about 10% of pretreatment values after T enanthate administration. A similar reduction in urinary epiT glucuronide excretion following administration of the same dose of T enanthate has previously been reported [16]. These authors also reported a reduction of urinary epiT sulfate excretion to about 15% of pretreatment values. However, based on direct measurements in spermatic venous and peripheral blood and on data on testicular blood flow, Dehennin [1] concluded that there is an extragonadal contribution of about 50% to epiT and 30% to epiT sulfate. The further metabolism of epiT and epiT sulfate secreted from the testis may make it difficult to draw conclusions about testicular secretion of these steroids from data on urinary excretion.

Despite the more pronounced increase in the urinary T/epiT ratio, this ratio was somewhat inferior to the serum T/17-OHP ratio as a marker of exogenous

T administration in the present investigation as well as in our previous short term study [9]. In the present study this was due to the anomalous behaviour of one single subject, whose urinary T/epiT ratio was below the upper reference limit at 3 and 9 months (Fig. 1). Although the basal androgen status of this subject was judged as normal from the serum T/SHBG ratio and LH and estrogen levels, he had the lowest serum T concentrations in the population at 0 and 3 months, the lowest urinary T/LH values at 0, 3 and 9 months and the lowest urinary T/Cr and T/epiT values during the entire period of observation. The possibility must be considered that this subject has some aberration in T metabolism, interfering with the urinary T excretion and T/epiT ratio. It should be pointed out that other markers of T administration, i.e. serum T/17-OHP and serum and urinary T/LH ratios followed the expected pattern.

Significant positive correlations were observed between serum T on one hand and urinary T/Cr and T/epiT ratio (r_s=0.56, P<0.05 and r_s=0.60, P<0.01, respectively) on the other. Furthermore, there were significant positive correlations between serum E_2 and tE_1 on one hand and urinary tE_1/Cr on the other (r_s=0.52, P<0.05 and r_s=0.78, P<0.001, respectively). There were, however, no correlations between serum and urinary LH. This may in part be explained by the low LH levels detected in most samples. In addition, small variations in renal function may affect urinary excretion of the larger LH molecule more than that of the steroids.

Although the levels of estrogens and SHBG changed significantly during treatment, they do not seem to be of value as markers of exogenous T administration. Their changes do not reflect direct effects on the pituitary–testicular axis. It is well known that administration of aromatizable androgens, such as T, results in increased estrogen levels [17]. Circulating concentrations of the most potent estrogen, E_2, and the serum T/SHBG ratio, which is an index of biologically active T, both increased by a factor of about 3. An identical relative increase of E_2 and T, although less pronounced, was also found in our previous short-term study [9]. The estrogen–androgen balance thus seems to be unaffected during T enanthate treatment. The modest decrease in circulating SHBG reflects the suppressive effect of T on hepatic SHBG output. However, we found no correlations whatsoever between SHBG and T, despite the significant changes in both following T enanthate administration. This may further support the hypothesis that the effects of endogenous or parenterally administered sex hormones on SHBG may be indirect and mediated by other factors [18].

The results of the present study support the contention that serum T/17-OHP ratios, serum and urinary LH concentrations, as well as T/LH ratios, are valuable markers of exogenous T administration in men. Thus, we suggest complementary immunological analysis of T, 17-OHP and LH in serum in all unclear male cases where T doping is suspected on the basis of an elevated T/epiT ratio in urine. Furthermore, in contrast to the urinary T/epiT ratio, these alternative markers will also indicate T doping in cases of simultaneous self-administration of T and epiT [19]. In cases where a positive result can have legal consequences, the T/17-OHP ratio has the advantage of verification by isotope dilution–mass spectrometry [20, 21].

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