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Testosterone Detection in Different Ethnic Groups.

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1. Introduction

The administration of exogenous testosterone produces changes in the urinary steroid profile. Based on studies by Donike et al. (1983), the International Olympic Committee (IOC) adopted in 1983 the ratio between testosterone (T) and its 17 α -epimer, epitestosterone (E), as a marker of testosterone abuse. A sample with a T/E ratio above 6 should be considered as positive to T administration. The application of such an approach presents some problematic aspects due to genetic, environmental or dietetic factors that may modify the ratio and conduct to false positive or negative cases.

Different approaches to overcome this situations had been proposed. The increase of blood plasma testosterone after the administration of exogenous testosterone (usually testosterone esters), produces the inhibition of the secretion of gonadotrophines at the hypophysary level (luteinizing hormone, LH) and a decrease in the production of the metabolic precursor of the endogenous testosterone (17 α -hydroxyprogesterone, 17OHP). Parameters as T/LH in urine or plasma (Kicman A.T., 1991) and T/17OHP in plasma (Carlström K., 1992, Palonek E., 1995) can be useful to disclose doubtful cases.

Testosterone is usually administered intramuscularly as different 17 β -hydroxy esters. Nearly complete hydrolysis of these testosterone-esters by plasmatic esterases give rise to the active testosterone. Nevertheless, minute amounts of unchanged esters remain in the body. The detection of the unchanged testosterone ester in human plasma would therefore provide an unambiguous confirmation of testosterone abuse. Some preliminary results on

one volunteer receiving testosterone intramuscularly or orally had been described (de la Torre X., 1995). In that study, the detection in blood plasma of the ester administered was possible over the same time range in which T/E was elevated in urine. Additional data with a larger number of volunteers appeared to be needed.

Ethnic differences on sex hormones levels in plasma or in urine had been established (Okamoto, 1971; Key, 1990; Ellis, 1992). Preliminary results (unpublished, Figure 1.) for the T/E in samples obtained during the Barcelona Olympic Games 1992 (Oriental athletes represent only a few percent of the population) show striking differences as compared to full population of athletes from Oriental origin. Accordingly, the steroid profile obtained after application of exogenous testosterone to different ethnic groups is expected to result in relevant differences among them. For this purpose, the response on the steroid profile in urine and plasma of Chinese and Caucasian volunteers after the intramuscular administration of a single 250 mg dose of testosterone enanthate was investigated under controlled conditions.

2. Experimental

2.1. Subjects and clinical protocol

Six healthy Caucasian (Spanish) males (25.5 ± 2.6 years, 72.7 ± 4.72 kg, 1.78 ± 0.04 m) (all values mean \pm sd) and eight healthy Oriental (Chinese, Han) males (19.8 ± 1.6 years, 64.6 ± 4.9 kg, 1.71 ± 0.05 m) volunteered for this study. The experiments with Caucasian and Oriental volunteers were performed respectively at the Municipal Institute for Medical Research (IMIM) of Barcelona, Spain and at The National Research Institute of Sports Medicine, China Doping Control Centre of Beijing, P.R. China.

All the subjects that volunteered for this study, received at time 0 h (usually 9.00 a.m.), 250 mg of testosterone enanthate (Testoviron Depot[®], Schering Germany, Batch # 31422) intramuscularly.

2.2. Sample collection

Plasma

10 mL de venous blood were obtained from the antecubital vein and collected in heparinized tubes. Immediately after the collection, the blood was centrifuged at 4°C and the plasma separated and kept frozen at -20°C until the analyses were performed.

Blood samples were collected at -72, -48 and -24 hours before the administration, at 8 and 24 hours along the next 5 days, and at 24 h at days 6, 7, 8, 10, 12 and 14 after the administration.

Urine

The first morning urine was collected at -72, -48 and -24 hours before the administration. From day 1 to 5, the whole 24 hours urine was collected in two portions, from 0-8 hours and 8-24 hours. Additional spot samples were obtained early in the morning at days 6, 7, 8, 10, 12 and 14 after the administration. The volumes of the samples obtained were registered and an aliquot of 50 mL was kept frozen at -20°C until the analyses were performed.

2.3. Reference steroids

Testosterone (T), 17 α -epitestosterone (E), androsterone (A), etiocholanolone (Et), dihydrotestosterone (DHT), 3 α ,5 α -androstane-3 α ,5 α -diol (5 α DIOL), 3 α ,5 β -androstane-3 α ,5 β -diol (5 β DIOL), 17 α -methyltestosterone (MT; internal standard), testosterone-17 β -enanthate (Te) and 17 α -hydroxyprogesterone (17OHP) were purchased from Sigma (St. Louis, USA). Testosterone-[16,16,17]-d₃ (Td₃), 17 α -epitestosterone-[16,16,17]-d₃ (Ed₃) and etiocholanolone-[2,2,4,4]-d₄ (Etd₄) were kindly provided by Prof. M. Donike (Deutsche Sporthochschule, Cologne, Germany).

2.4. Sample preparation

Plasma

Testosterone enanthate (Te), testosterone (T) and 17 α -hydroxyprogesterone (17OHP) were

analysed by gas chromatography/mass spectrometry (GC/MS) and luteinizing hormone (LH) was determined by Microparticle Enzyme Immuno Assay (MEIA) (Abbott Laboratories, Ill, USA).

A previously described method (de la Torre X., 1995) was used for plasma sample extraction. Briefly, frozen plasma samples were allowed to stand at room temperature for thawing and then kept in an ice-bath. MT was added, at a final concentration of 4 ng/mL, as internal standard. Immediately after subsequent addition of 50 μ l of 3 M potassium hydroxide, the samples were extracted at 4°C twice with 4 mL of a n-hexane:ethyl acetate (70/30) mixture using a rocking mixer for 20 min. The organic phases were pipetted out in a tube, mixed and washed with 1 mL of 5% acetic acid and then with 1 mL of distilled water. After evaporating the solvent mixture under a nitrogen stream, the residue was kept in a dessiccator over potassium hydroxide and phosphorous pentoxide for at least 30 min. before derivatization (see below).

Quantitative analyses of testosterone enanthate were performed by GC/MSD using a calibration curve in duplicate prepared by adding known amounts of testosterone enanthate to 1 mL of plasma at a final concentration of 0.2, 0.4, 0.8, 2, 4 and 8 ng/mL. The quantitation of free testosterone and 17-hydroxyprogesterone by GC/MSD was based on the response factor calculated from extracted samples from distilled water, containing 4 and 8 ng/mL of testosterone and 0.5 and 1 ng/mL of 17OHP. Calibration samples were extracted as real plasma samples.

LH by MEIA was analysed directly in untreated serum using the controls provided and following manufacturer's protocols.

Urine

Testosterone (T), 17 α -testosterone (epitestosterone, E), and other parameters of steroid profile were analysed by GC/MS according to the routine screening procedure.

2.5 mL of urine were extracted in XAD-2 columns (Biochemical Diagnostics Inc., NY, USA) previously washed with methanol and water. Steroids were eluted with methanol, the solvent evaporated and the hydrolysis was carried out with 5000 IU of β -glucuronidase

from *E. coli* (K12) in 1 mL of phosphate buffer 0.2 M, pH=7 for 1 hour at 55°C. After cooling, the pH was adjusted to 9-10 and steroids were extracted with 5 mL of diethyl ether. The organic solvent was taken to dryness and the samples kept in a dessiccator for at least 30 min. before derivatization.

Quantitation of the endogenous steroids:

In each analytical batch, a calibration methanolic extract was injected to calculate response factors to quantify each substance. Concentrations of the substances in the calibration mixture are summarised in Table 1.

2.5. GC/MSD analysis

Derivatization: For the trimethylsilylation (TMS) of steroids in both urine or plasma extracts, the dry residues were dissolved in 20-50 μ L of MSTFA/ NH_4I /Dithioerythritol (1000:2:5) and heated at 60°C for 15 minutes. 2-3 μ L of each solution were directly injected into the GC-MSD system.

Plasma

A gas chromatograph model 5890 series II (Hewlett-Packard (HP), Palo Alto, CA) fitted with a model 7673A autosampler was connected to a mass selective detector 5970 (HP). The separation was carried out using a 5% phenylmethylsilicone fused silica capillary column (HP5 Ultra 2, 12 m, 0.2 mm i.d., film thickness 0,33 μ m).

The injector, operated in splitless mode (0.3 min.) and the interface were maintained both at 280°C. The oven temperature program was: initial temperature 190°C (0.5 min.), *rate 1*: 30°C/min to 280°C, *rate 2*: 40 °C/min to 310°C, and maintained for 3.50 min at 310°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min at 180°C. Mass spectra of reference standards were obtained in scan mode. For the analyses of plasma samples, the mass spectrometer was operated in the Selected Ion Monitoring (SIM) acquisition mode, only the molecular ions (Table 2) being selected for each compound.

Urine

A gas chromatograph model 5890 series II (Hewlett-Packard (HP), Palo Alto, CA) fitted with a model 7673A autosampler was connected to a mass selective detector 5971A (HP). The separation was carried out using a methylsilicone fused silica capillary column (HP1 Ultra 1, 17 m, 0.2 mm i.d., film thickness 0,11 μm).

The injector, operated in split mode (1:10 split ratio) and the interface were maintained both at 280°C. The oven temperature program was: initial temperature 180°C, *rate 1*: 3.3°C/min to 231°C, *rate 2*: 30 °C/min to 310°C, and maintained for 2 min at 310°C. Helium was used as a carrier gas at a flow rate of 0.8 mL/min at 180°C. For the urine analysis, the mass spectrometer was operated in the Selected Ion Monitoring (SIM) acquisition mode, one or two ions being selected for each compound (Table 1).

Table 1. Absolute retention times, selected ions for each analyte-TMS derivative (urine analysis) and concentrations in the calibration mixture.

Compound	RT	Conc. (ng/mL urine)	m/z
Androsterone (A)	10.97	2000	434
Etiocholanolone-d4 (Etd4)	11.00	500	438
Etiocholanolone (Et)	11.14	2000	434
3 α ,5 α -androstanediol (5 α DIOL)	11.26	80	241
3 α ,5 β -androstanediol (5 β DIOL)	11.38	120	241
Epitestosterone-d3 (Ed3)	12.60	15	435
Epitestosterone (E)	12.65	20	432
Dihydrotestosterone (DHT)	12.72	40	434, 143
Testosterone-d3 (Td3)	13.46	90	435
Testosterone (T)	13.50	120	432
Methyltestosterone (MT) <i>ISTD</i>	15.18	500	446

Table 2. Retention times and selected ions for each analyte TMS derivative for plasma analysis.

Compound	RT	RRT	m/z
Testosterone (T)	4.56	0.944	432
Methyltestosterone (MT) <i>ISTD</i>	4.83	1.000	446
17 α -hydroxyprogesterone (17OHP)	5.51	1.141	546
Testosterone enanthate (Te)	6.76	1.400	472

3. Results and discussion

Blood plasma

Plasma basal testosterone levels in the Orientals were significantly lower than those presented by the Caucasians before the ester administration. Plasma levels observed in the Caucasian group are in agreement with previously published values (Schutle-Beerbühl M., 1980; Schürmeyer Th. and Nieschlag E., 1984; Behre H.M., 1990). Differences were not observed in basal levels of LH or 17OHP.

Significant differences were observed on the pharmacokinetic profiles of testosterone enanthate and testosterone after the administration in the two groups studied.

Testosterone enanthate.

As it can be seen in Figure 2. there are differences in the absorption rates (T_{max} and C_{max}) and excretion rates ($t_{1/2}$) in the two groups studied (de la Torre X., 1995).

Testosterone enanthate could be detected in plasma over 10-11 days in the Caucasian group and during 7-8 days among Orientals.

Testosterone.

Differences observed in the plasma testosterone levels were a direct consequence of the testosterone enanthate pharmacokinetics. After testosterone enanthate administration, the hydrolysis of the ester produces an increase of testosterone plasma concentrations (Baba S., 1980).

In the Oriental volunteers both absorption and excretion were faster leading to high concentration peaks at shorter kinetic times (figure 3). The maximum concentration reached is similar to the one presented by the Caucasians but because the initial levels were lower in the Orientals, the relative increase compared to the concentrations before the administration is more important in the latter (figure 4).

After the administration of testosterone esters, two phenomena are observed: the inhibition of the endogenous synthesis of testosterone and simultaneously the release of the exogenous

testosterone in the blood stream. The decrease, under the basal levels, of testosterone concentrations observed in days 10-11 (6-7 in the Orientals) after the administration had been described previously (Herman M Behre i col. 1990) in primary hipogonadal patients receiving testosterone and is due to the maintenance of the gonadal inhibition of the endogenous testosterone synthesis. At the end of the time studied (14 days after the administration), the Orientals had nearly recovered the basal levels prior to the administration while the Caucasians still had the concentrations depressed.

Effects on the hypothalamic-hypophysary-gonadal (HHG) axis.

The exogenous testosterone or anabolic steroids administration leads to an inhibition of the HHG axis, more or less marked depending on the substance abused, the dose and the kind of administration. The inhibition can be at the hypothalamic-hypophysary level and/or at gonadal level.

As was expected (Alén M., 1985; Ruukonen A., 1985) due to the inhibition of the hypothalamic-hypophysary-gonadal axis, a marked decrease in the plasmatic gonadotrophines (LH) (figure 5) and in the metabolic precursor of testosterone (17OHP) (figure 6) was observed in all the volunteers.

Simultaneously, the increase of testosterone concentrations (of exogenous origin) and the decrease in LH and 17OHP, leds to a significant increase of the T/LH (figure 7) and of T/17OHP (figure 8) ratios as has been described previously (Kicman A.T., 1991; Cälstrom K., 1992).

Urine

Significant differences among Caucasians and Orientals regarding basal testosterone glucuronide excretion in urine were observed but not in the case of the excretion of its 17 α -epimer (epitestosterone).

After testosterone enantate administration, the relative increase in urinary testosterone excretion was higher in Orientals, but absolute concentrations never reached basal

Caucasians levels. On the other hand, the decrease of epitestosterone concentrations was similar in both groups, in absolute values or in relative values to basal levels.

Effect on the T/E ratio.

The determination of testosterone abuse by Sport Authorities is based on the urinary testosterone to epitestosterone ratio (T/E). After an exogenous testosterone administration, there is an inhibition of endogenous steroids synthesis and a decrease of epitestosterone excretion is observed. Simultaneously, testosterone excretion in urine is increased and then we can detect a significant increase of the T/E ratio.

Based on population studies of the urinary T/E ratio (Donike et al., 1983) the Medical Commission of the IOC established in 1983 that a urine sample showing a T/E ratio higher than 6, should be considered as positive to the abuse of testosterone.

Our results have shown that basal testosterone glucuronide concentrations are significantly lower in Orientals while epitestosterone glucuronide values are quite similar. This leads to basal T/E significantly different, being in the Orientals about 1/5 of the Caucasians values (figure 1).

As expected in the Caucasians group, the administration of exogenous testosterone leads to a fast increase of the T/E in urine above the regulatory cut-off of 6, being the effect observed along the next 10 days after the administration (figure 9). In the Orientals we should distinguish two different groups of volunteers. Two of the eight subjects showed T/E basal ratios similar to those presented by the Caucasians and their behaviour after the administration was as described before. The other 6 Chinese volunteers had very low basal values of the ratio (in agreement with the expected population distribution of Orientals), and even if the relative increase of the ratio was higher compared to the other subjects, in these group they never reached the value of 6 giving a maximum value around 4. The samples of these subjects should be considered negatives to testosterone administration according to the actual regulations (figure 10).

The population values used to establish up to date the normal population ranges, were obtained mainly from samples of Caucasian population. Data obtained from antidoping

laboratories in Asia suggested that Orientals present lower T/E values than Caucasians (Park J. i col, 1990). This point was also observed in the T/E distribution of the samples analysed during the 1992 Olympic Games in Barcelona. Samples of oriental athletes were in the lower range of the distribution.

An interesting point was to describe how the basal differences in the T/E ratios in both populations studied could influence to an exogenous testosterone administration.

The criterion based on population studies ($T/E > 6$) is not useful to detect a single testosterone administration in most of the Chinese volunteers and very probably in subjects with a low basal T/E ratio, independently of their ethnic origin. Other blood plasma alternative criteria (T/LH, T/17OHP) seem to have more diagnostic possibilities for long term detection, even if different cut-off points should be establish for every population group.

The detectability of testosterone esters in plasma in the same time period in which T/E is altered in Caucasians has already been demonstrated (de la Torre X., 1995). There are pharmacokinetic differences in T enanthate excretion between the 2 groups studied. In Orientals the detection of the ester was possible even in those cases where the urinary T/E ratio did not reach the IOC limit of 6.

4. Conclusions

Different urinary steroid profile (absolute concentrations and ratios) and different pharmacokinetic parameters in plasma were observed between Chinese and Caucasians after the administration of testosterone enanthate. Differences were also found in the basal levels.

In individuals with low basal T/E, criteria for positiveness based on longitudinal studies should replace population based ones.

Ethnic differences should be considered in hormone replacement therapies, use of testosterone esters in male birth control programs and for a correct interpretation of athletic doping control tests.

5. Acknowledgements

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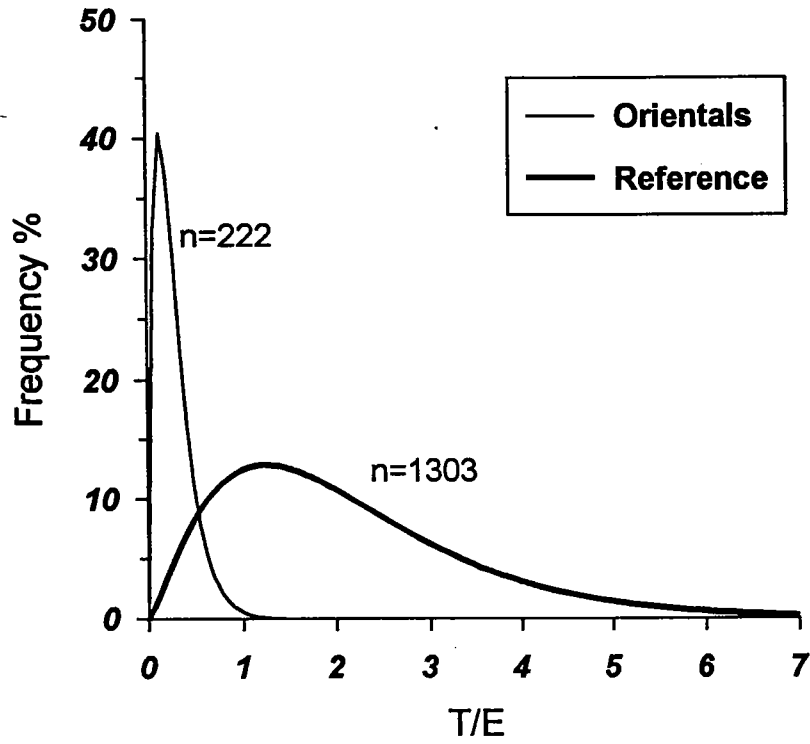


Figure 1. Urinary males T/E ratio frequency distributions in Orientals (n=222, from the database of China Doping Control Centre, Beijing) and Reference values obtained in the Barcelona Olympics Games 1992 (n=1303) .

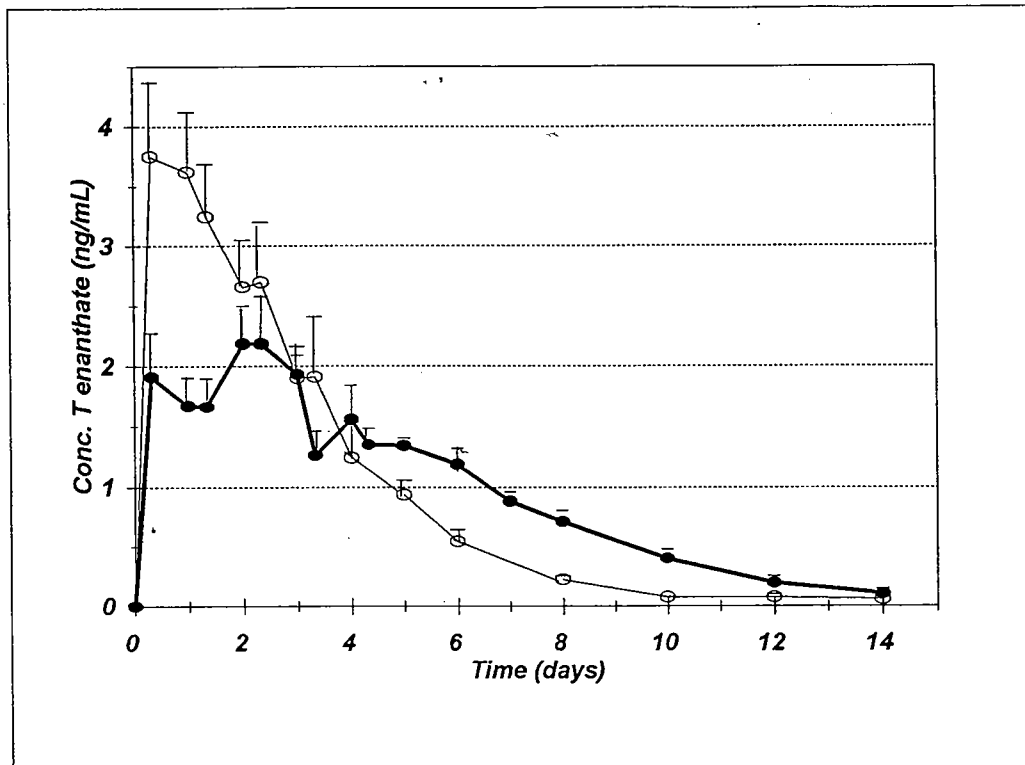


Figure 2. Plasma T enanthate concentrations in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0 (mean±SEM).

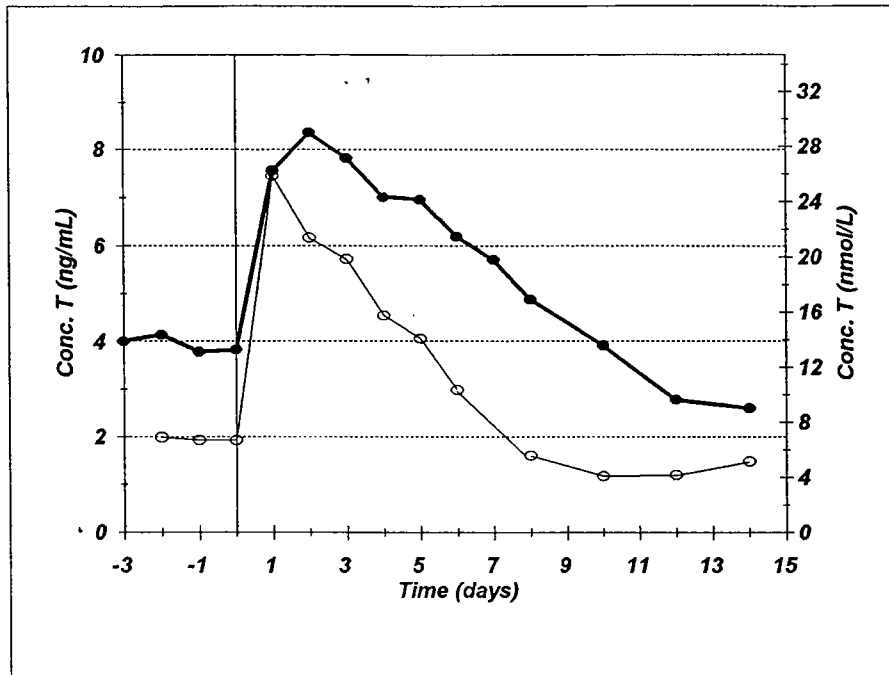


Figure 3. Plasma T concentrations in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

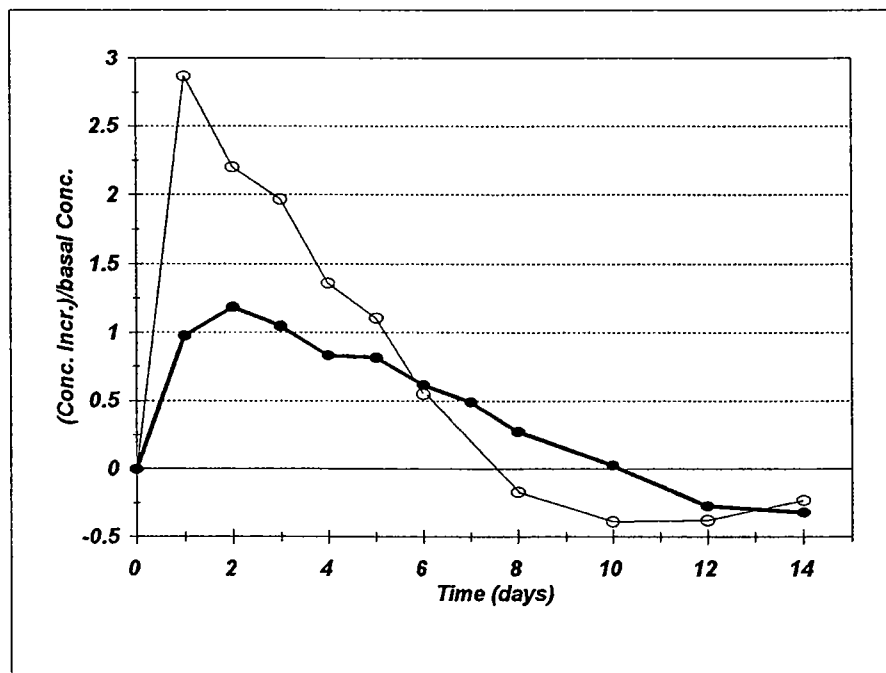


Figure 4. Relative plasma T concentrations in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

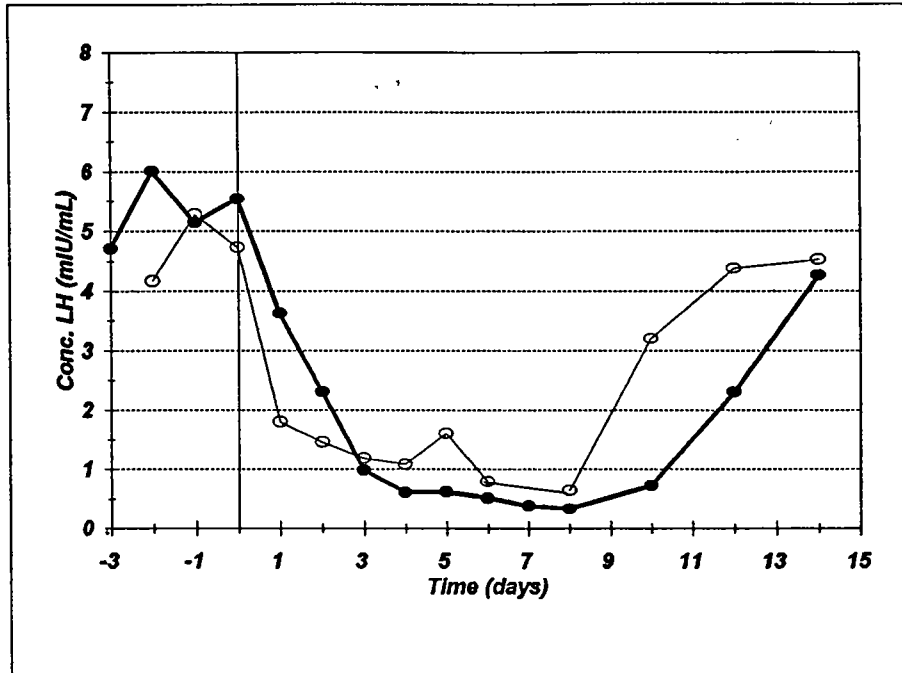


Figure 5. Plasma LH concentrations in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

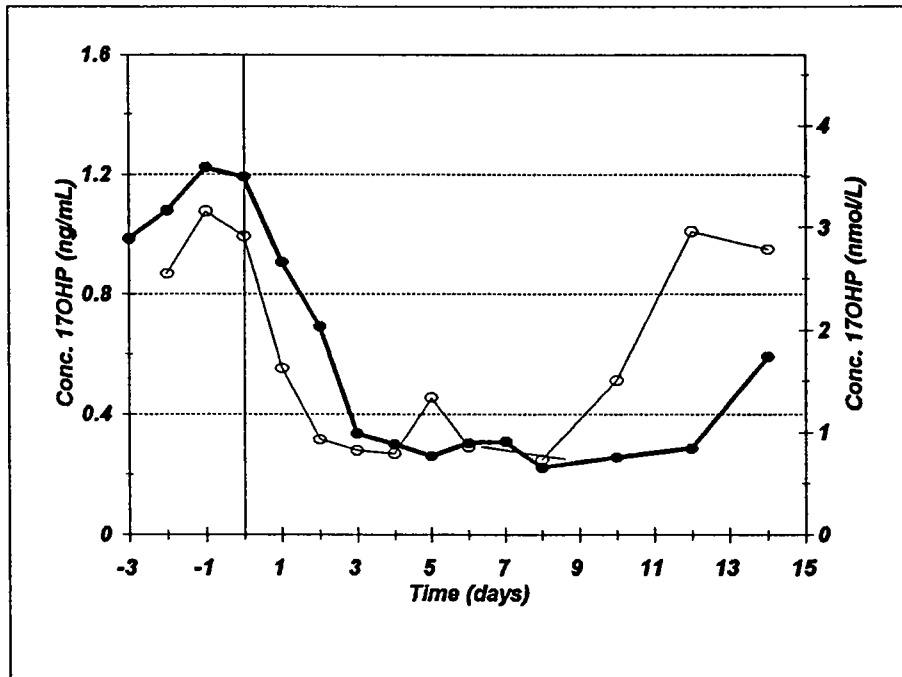


Figure 6. Plasma 17OHP concentrations in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

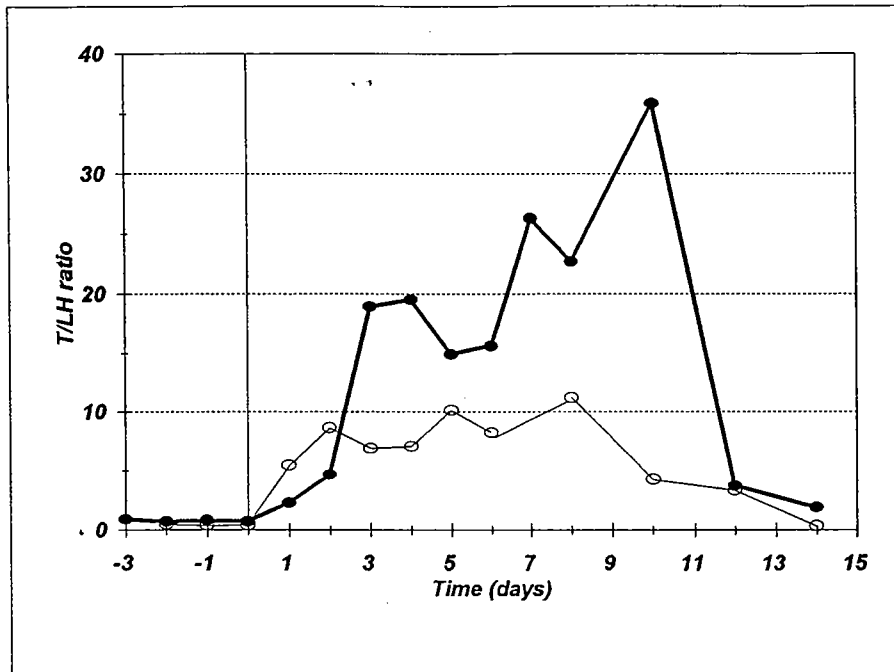


Figure 7. Plasma T/LH ratio in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

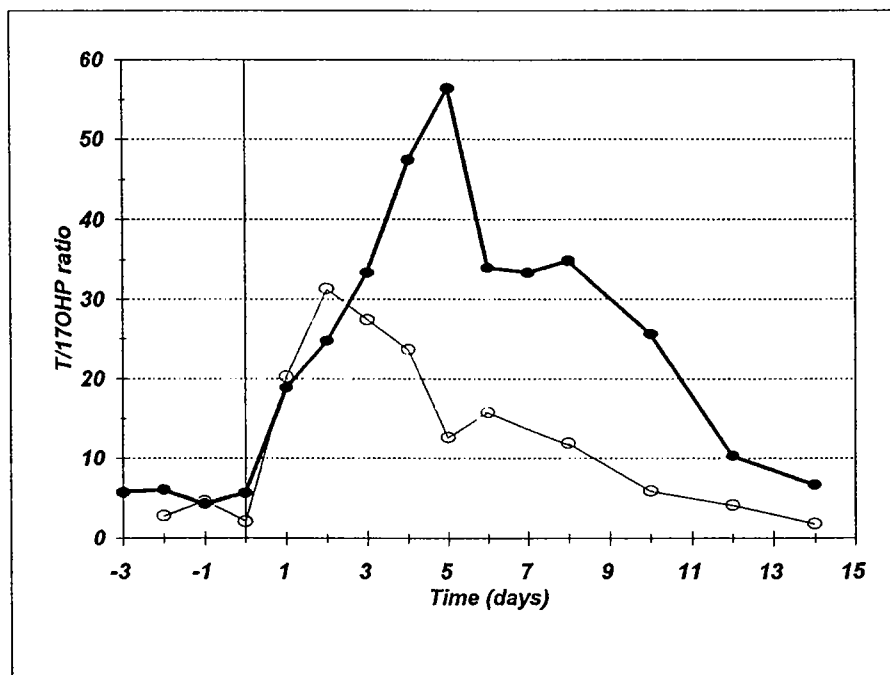


Figure 8. Plasma T/17OHP ratio in Caucasians (n=6) (●) and Orientals (n=8) (○) after 250 mg of testosterone enanthate intramuscular administration at time 0.

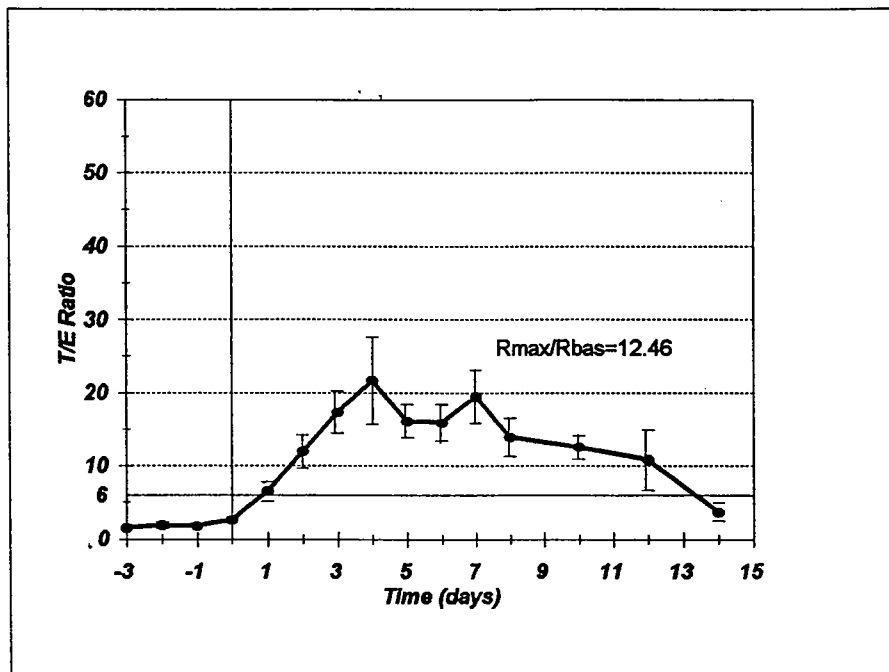


Figure 9. Urinary T/E ratio in Caucasians (n=6) (●) after 250 mg of testosterone enanthate intramuscular administration at time 0 (mean±SEM).

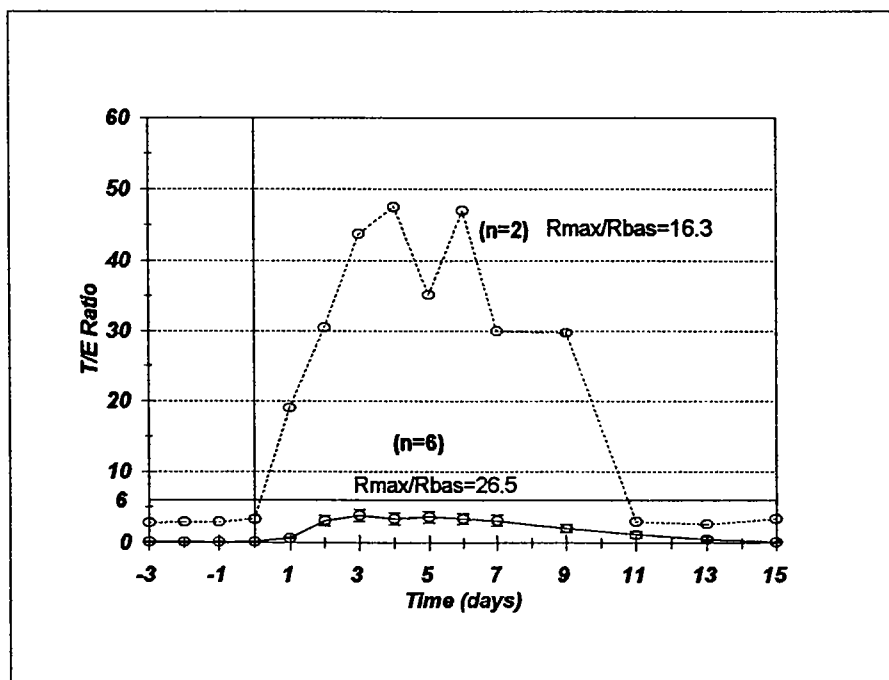


Figure 10. Urinary T/E ratio in Orientals with low basal T excretion (n=6) (—) and high basal T excretion (---) after 250 mg of testosterone enanthate intramuscular administration at time 0 (mean±SEM).