Criteria to indicate testosterone administration

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A detection method for testosterone administration was developed using radioimmunoassay to measure the urinary ratios of testosterone (T) to epitestosterone (E) and to luteinizing hormone (LH). A comparative study of the effect on these ratios of a single intramuscular injection of testosterone heptanoate followed by stimulation with human chorionic gonadotrophin (HCG) in three normal men was undertaken. To allow immediate investigation, a commercially supplied epitestosterone antiserum was used. This study showed that both T/E and T/LH ratios could be used to detect testosterone administration, the latter also being an indicator of HCG use due to cross-reactivity with the LH antiserum. Subsequently, an epitestosterone antiserum of superior specificity was raised and used in a study to demonstrate the insignificant effect of exercise on these ratios. Finally, an intramuscular injection of a combined preparation of testosterone/epitestosterone heptanoates resulted in raised ratios of T/LH but not of T/E. This demonstrated the importance of the T/LH ratio in circumstances where the T/E ratio can be easily circumvented.

Keywords: Testosterone, epitestosterone, luteinizing hormone, human chorionic gonadotrophin

Introduction

Detection of administration of the naturally occurring anabolic steroid testosterone presented an entirely new problem for drug control in sport. Hitherto, only foreign substances had been banned and all that was required to indicate use was unequivocal proof that a banned substance and/or its metabolites were present in a urine sample. Since untimed urine samples are collected from athletes, which vary considerably in concentration, it is not possible to set a limit for the normal level of excretion of urinary testosterone. In 1979 Brooks *et al.* investigated the possibility of detecting testosterone abuse by its feedback effect on the suppression of luteinizing hormone (LH)[1]. The ratio of total testosterone to LH (T/LH) in the urine was found to increase after an intramuscular injection of testosterone heptanoate in all ten normal males, although there was an overlap between the basal range and that obtained three days after injection. The method used was radioimmunoassay as protein hormones cannot be measured by gas liquid chromatography-mass spectrometry (GLC-MS).

In 1983, the International Olympic Committee (IOC) adopted the ratio of urinary testosterone to epitestosterone (T/E) as the sole test for testosterone doping as both these hormones could be conveniently measured by GLC-MS.

Epitestosterone is the inactive 17α-epimer of testosterone which is also secreted by the gonads. Male or female urine contains approximately equal concentrations of testosterone and epitestosterone[2]. Donike *et al.* showed that the average ratio of urinary testosterone to epitestosterone in 50 normal males was 1.13 ± 0.57 SD (range 0.12 to 4.44) and in 47 normal females was 1.29 ± 0.89 SD (range 0.26 to 2.90) using combined GLC-MS techniques[3]. As epitestosterone is only a very minor product of the metabolism of testosterone, androstenedione or dehydroepiandrosterone[3–5], the detection of testosterone doping can be determined by an increase in the urinary T/E ratio.

The initial aim of this study was to develop a radioimmunoassay as an additional rapid screening technique for assessing urinary T/E ratios in athletes. Kits could then be supplied to laboratories in countries which did not have GLC-MS facilities. The methods for determining urinary T/LH ratios were also improved for two reasons. First, the grounds for banning a sports individual using testosterone would be stronger if two relevant urinary indices are used instead of one. Secondly, the use of the T/LH ratio for screening for testosterone administration may be a better method for the future. Although the IOC adopted the use of the T/E ratio, it is feasible that this test may be circumvented. Since only approximately 1% of testosterone is excreted unchanged (apart from being conjugated with glucuronic acid) compared with 30% of epitestosterone, an intramuscular administration of these two respective hormones in a ratio of approximately 30 to 1 may give a normal urinary ratio of 1. Alternatively, human chorionic

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0306-3674/90/040253–12

gonadotrophin (HCG) could be administered to male athletes to stimulate endogenous testosterone and epitestosterone production, thus raising 'beneficially' the blood testosterone concentration without altering the urinary T/E ratio.

To allow immediate investigation, a commercial epitestosterone antiserum was purchased. The normal ranges of T/E and T/LH ratios in male urine together with cut-off points were established. The effect of a single dose of testosterone heptanoate followed by HCG stimulation on these urinary ratios and selected serum hormones was studied in three normal men. Urinary HCG was measured after HCG stimulation by means of a commercial enzymatic immunoassay kit and a specific radioimmunoassay (routinely performed by the Supraregional Assay Laboratory at Charing Cross Hospital, London).

Subsequently, a more specific epitestosterone antiserum was raised. During this period the National Institute for Biological Standards and Controls stopped supplying human urinary menopausal gonadotrophin standard and recommended the use of pituitary LH standard for urinary LH assays. New normal ranges of T/E and T/LH ratios were evaluated. The effects of strenuous exercise on these ratios were investigated to establish whether any changes may give rise to false positives.

Finally, a male volunteer who had participated in the previous experiment (testosterone/HCG administration) was injected intramuscularly with a combined preparation of testosterone heptanoate with epitestosterone heptanoate in a ratio of 28:1. The effects on the urinary T/E and T/LH ratios were studied in the hope of illustrating the importance of the latter ratio where the use of the T/E ratio may be negated.

Experimental materials

Antisera

The testosterone antiserum produced in our laboratory and the commercially supplied epitestosterone antiserum (obtained from Wien Laboratories, Succasunna, NJ 07876, USA) were both raised to their respective steroid-3-(0-carboxymethyl)oxime-protein conjugate.

Epitestosterone antiserum was raised against epitestosterone-3-(0-carboxymethyl)oxime bound to bovine serum albumin by the method of Erlanger *et al.*[6]. The immunogen was injected subcutaneously into a 'New Zealand White' rabbit. The antiserum was harvested after six months.

The LH antiserum was kindly supplied by Professor W. Butt of the Birmingham and Midland Hospital for Women, Birmingham. Gonadotrophin reference preparations were kindly supplied by Dr P. Storring of the National Institute for Biological Standards and Controls, Hertfordshire.

Radioiodinated labels

Testosterone and epitestosterone ^{125}I-radiolabels were prepared by the method of Nars and Hunter[7].

Radiolabelled ^{125}I-LH was prepared using pituitary LH standard (2 μg, IRP 2/69) by a modification of a method for radiolabelling human growth hormone[8].

Assay buffer

The buffer used was NAFA as described by Brooks *et al.*[9] with bovine serum albumin (BSA, 0.1–2.5% w/v).

Methodology

The measurement of total urinary testosterone and epitestosterone

Urinary steroids were hydrolysed with glucuronidase, extracted and measured by radioimmunoassay.

Male urine (25 μl) was diluted with distilled water (500 μl) and 550 Fishman units of β-glucuronidase enzyme[10] in acetate buffer (50 μl, 1M, pH 4.5) was added. The tubes were sealed and incubated at 55°C for one hour.

The hydrolysed samples were then cooled in an ice bath. Extraction was performed by the addition of dichloromethane (4 ml) and shaking for 10 minutes on a multivortex mixer.

The tubes were then returned to the ice bath and the upper aqueous layer removed by suction. Portions (1 ml) of the extracted samples were dispensed into glass tubes and dried down under air at 37°C.

The dried extracts were reconstituted with NAFA buffer (0.1% bovine serum albumin, 2.5 ml). The reconstituted sample extracts were used for both the total urinary testosterone and epitestosterone in separate radioimmunoassays. Up to 35 samples may be analysed in one assay. Portions (100 μl) of the appropriate standards in assay buffer (0.1%BSA) were taken to give a calibration range of 0–200 pg/tube for the testosterone and epitestosterone assays.

Duplicate portions (100 μl) of standards and reconstituted sample extracts were dispensed into disposable polystyrene tubes (75 mm × 12 mm). Duplicate zero tubes (no standard present) and non-specific binding tubes (used to determine the fraction of labelled analyte which is bound to sites other than on the antiserum) were prepared using 100 μl and 200 μl assay buffer respectively.

The assays were incubated throughout at 4°C, adding precooled reagents with vortex mixing.

The appropriate antiserum was diluted to a working concentration with assay buffer. Testosterone antiserum was diluted 1:16K; the commercial epitestosterone antiserum was reconstituted with 30 ml assay buffer and our own laboratory raised epitestosterone antiserum diluted 1:16K. Portions (100 μl) were added to all the tubes except the non-specific binding tubes. The assay was incubated with antiserum for 30 minutes.

The relevant radioiodinated labelled steroid was diluted with assay buffer and portions (100 μl, 15000 cpm) were added to all the tubes. The assay was left to incubate for a further 60 minutes. Two total count tubes were also prepared containing label only for counting at the end of the assay. The antiserum bound steroid was then separated from the free using

an accelerated second antibody technique which included a wash step to lower the non-specific binding[11].

Polyethylene glycol (8%, w/v) was dissolved in assay buffer and mixed with second antibody (donkey – antirabbit, 1%, v/v) and rabbit serum (0.1%, v/v). The mixed suspension (500 µl) was added to all the tubes, incubated for 10 minutes and then centrifuged for 15 minutes at 1600 g and 4°C.

All but 100 µl of the supernatant was removed by suction using a Pasteur pipette fitted with a rubber collar to limit its penetration into the tubes.

Assay buffer (1 ml) with a higher protein concentration (2.5%, w/v bovine serum albumin) was added to the tubes and gently mixed to resuspend the pellet. Centrifugation was repeated with the same conditions as before and all the supernatant was removed by suction. The pellets (bound fraction) and the total tubes were counted.

Procedure for assaying LH in urine

LH was extracted by means of protein precipitation and then measured by radioimmunoassay using ^{125}I-labelled LH. This was a modified procedure of that described by Wheeler *et al.*[12]. Urine samples were adjusted to a pH between 4.5 and 6.5 using acetic acid (10%, v/v) and sodium hydroxide solution (0.1 M). Portions of urine (1 ml) were dispensed into plastic tubes (100 mm × 15 mm).

One drop of bovine serum albumin solution (30%, w/v) was added to each tube and then mixed. Ethanol (5 ml) was then added and the tubes were capped. The samples were mixed and left to incubate for one hour at 4°C with occasional mixing.

The tubes were then centrifuged (700 g, 4°C) for 10 minutes. The ethanol was discarded and the tubes left to drain for five minutes.

To each tube 1 ml of buffer (NAFA, no albumin added) and one drop of acetic acid (about 50 µl 10%, v/v) was added. The pellets were redissolved by mixing vigorously.

Ethanol (5 ml) was again added and the procedure repeated as before. The pellets were left to dry for one hour at room temperature to ensure that virtually all the ethanol had evaporated before the addition of buffer (NAFA, 1 ml, no albumin added). The pellets were dissolved in the buffer by gentle mixing for one hour at room temperature or by leaving them overnight at 4°C.

The radioimmunoassay for LH was performed as described. A working standard solution of either 2nd IRP Human Menopausal Urinary Gonadotrophins A60/25 or 1st IRP Human Pituitary LH 68/40 was prepared in assay buffer (NAFA containing BSA, 2.5% w/v) to give a value of 40 International Units (IU)/100 ml or 19.2 IU/100 ml respectively. Portions (500 µl) were diluted to give a range of 400 mIU/ml to 3 mIU/ml (2nd IRP A60/25) or 192 mIU/ml to 0.75 mIU/ml (1st IRP 68/40).

Duplicate portions (100 µl) of the diluted standards and extracted samples (up to a maximum of 80 samples) were dispensed into polystyrene tubes (75 mm × 12 mm). Duplicate zero tubes and non-specific binding tubes were prepared using 100 µl and 350 µl of assay buffer, respectively.

LH antiserum was diluted using assay buffer (1:800K) to give approximately 35% of the counts obtained from the antiserum bound fraction with no analyte present compared with the total counts from the tracer alone (B_0/T). The diluted antiserum (250 µl) was added to all the tubes except the non-specific binding tubes and incubated for six hours at room temperature.

Diluted LH tracer was then added to all the tubes (100 µl, 15000 cpm) and the assay incubated overnight at 4°C. Two total count tubes containing label only were prepared for counting at the end of the assay. The separation technique was the same as that used for the testosterone/epitestosterone method but the wash step was omitted and the second antibody solution was left to incubate for one hour at room temperature instead of 10 minutes at 4°C.

Counting of radioimmunoassays and calculation of results

All assays were counted on an LKB Multigamma 1260 counter for 100 s. The duplicate tube counts were averaged and corrected for non-specific binding. Calibration curves of percentage bound of the zero counts (B/B_0) versus the concentration of the standards were constructed on log-linear graph paper. The concentrations of the samples were interpolated from the curve.

The results for the testosterone and epitestosterone assay were expressed in nmol/l (nmol/l = 13.9 × pg/tube). Hence the ratios of testosterone to epitestosterone (T/E) were expressed as a number with no units.

The results for the LH assay were calculated in International Units per litre (IU/l) by multiplying the results interpolated from the curve by 10. The ratios of testosterone to LH were expressed in nmol/IU.

Normal ranges, drug administration and effect of exercise

Normal ranges of T/E and T/LH ratios in male urine were determined by using commercially supplied epitestosterone antiserum and menopausal urinary gonadotrophin standard. Single random urines were collected from 61 male medical students for T/E evaluation and 113 male students for T/LH evaluation. The samples were stored at 4°C until analysed.

Changes in urinary T/E and T/LH ratios and selected plasma hormones and SHBG in response to testosterone heptanoate administration followed by HCG stimulation were also assessed. The volunteers were three healthy males, subjects 1, 2 and 3, aged between 23 and 25 years. Informed consent was obtained.

A single intramuscular dose of testosterone heptanoate (Primoteston®, 250 mg) was administered at 11.00 h on day 1 of the investigation followed by HCG stimulation (5000 units, i.m.) at 15.00 h on day 15.

Blood samples were collected between 11.30 and

12.00 h on days 1 to 5, 8, 12, 15 to 19, 23 and 26. In one subject, days 8 and 12 were omitted due to problems of collection.

Urine samples were collected from days 0 to 19, 21, 23, 26 and 28 either as collections between 09.00 and 11.00 h or by 24 h collections ending before 11.00 h for days 0 to 1, 2 to 3 and 7 to 8 (subject 2 random collections day 21 and 28 omitted, subject 3 day 26 omitted).

Serum concentrations of testosterone, LH, follicle stimulating hormone (FSH), oestradiol, prolactin and sex hormone binding globulin (SHBG) were measured by radioimmunoassay in the routine hormone laboratory at St. Thomas' Hospital.

Total urinary testosterone, epitestosterone and LH were measured as described using the commercially supplied epitestosterone antiserum and the urinary gonadotrophin standard.

Urinary HCG was measured on days 15 to 17, 19, 21, 23 and 26 using the Model Plus Kit obtained from Alpha Laboratories, Eastleigh, Hants. The kit uses monoclonal antibody-based enzyme immunoassay for the qualitative detection of HCG in pregnancy, giving a blue colour for positive samples. The kit was modified by sampling 200 µl urine instead of the recommended 50 µl with the aim of ensuring an 'all or nothing' test. Qualitative results were determined visually by comparing the colour development of the sample to that of the positive quality control containing 50 IU/l of HCG. Sixty male medical students were negative when assayed for HCG, i.e. the samples exhibited no blue colour when assayed (less than approximately 25 IU/l).

The urine samples were then assayed for HCG using a specific radioimmunoassay in the Supra-regional Assay Service Laboratory, Charing Cross Hospital, London.

Normal ranges of T/E and T/LH ratios were determined in male urine using our own laboratory epitestosterone antiserum and 1st IRP pituitary LH standard. At 30% displacement of the total radioligand bound, the sum of the cross-reactions of androsterone and aetiocholanolone was 0.14% with our own laboratory raised antiserum, as opposed to 0.91% with the commercially supplied antiserum. The use of the commercially supplied antiserum may have elevated the epitestosterone assay results considerably and therefore new urinary T/E ratios were determined in 40 male medical students who had not been previously analysed.

T/LH ratios were determined using 1st International Reference Preparation Human Pituitary LH standard 68/40 as the 2nd IRP Human Menopausal Urinary Gonadotrophin Standard was no longer available. This new reference preparation is stated to be considerably purer both immunologically and biologically. Thirty nine male medical students were assayed for urinary T/LH ratios (not previously analysed).

The effect of two hour non-stop vigorous exercise on the urinary T/E and T/LH ratios was studied. The first urine pass before and after a workout for nine competitive male members (22 to 37 years old) of the ADI Karate Club (Lambeth, London) were collected and analysed.

Epitestosterone heptanoate was synthesized[13] and the product was purified by means of counter current extraction followed by alumina column chromatography and finally by thin layer chromatography. Testosterone heptanoate (Primoteston®, 250 mg) together with the prepared epitestosterone heptanoate was dissolved in sesame oil (1 ml) in an attempt to prepare a mixture of the esters in a ratio of 30:1 (w/w). The preparation was checked for purity using high pressure liquid chromatography and GLC (G.J. Southan, Ph.D., University of London 1990) and found to have a ratio of esters of 28:1 (w/w).

The mixed preparation was then administered intramuscularly to a healthy adult male, after obtaining his informed consent, who had participated in the previous experiment of testosterone heptanoate/HCG administration (subject 2). Urine was collected for one day prior and 10 days following administration and the T/E and T/LH ratios were determined. Finally, the urines were analysed to determine the T/E ratios using GLC-MS and the results were compared with those obtained by radioimmunoassay.

Table 1. Assay validations using testosterone antiserum, commercially supplied epitestosterone antiserum and own laboratory epitestosterone antiserum

	Sensitivity[1] (pg/tube)	*Inter-assay imprecision* (% CV)	*Cross-reaction of steroids at 30% displacement of total radioligand bound* (%)			
			Testosterone	*Epitestosterone*	*Androsterone*	*Aetiocholanolone*
Testosterone	6.8	12.0[2]	n/a	0.53	0.18	<0.1
Epitestosterone (commercially supplied antiserum)	3.9	13.5[3]	1.06	n/a	0.53	0.38
Epitestosterone	6.8	13.3[4]	0.15	n/a	0.08	0.06

Key: n/a = not applicable
(1) Sensitivity calculated from 2 SD of mean counts of 16 replicates of the zero point
Range of quality controls: (2) 27 to 175 pg/tube
(3) 28 to 54 pg/tube
(4) 20 pg/tube

Results

Assay validations

The sensitivity, imprecision and specificity of the testosterone and epitestosterone assays are displayed in *Table 1*.

The relative standard deviation (percent CV) of the LH inter-assay imprecision for normal male quality controls using 2nd IRP Urinary Human Menopausal Gonadotrophin Standard and 1st IRP Human Pituitary LH Standard was 16.8% and 7.6%, respectively.

Normal ranges of T/E and T/LH ratios in male urine were determined by using commercially supplied epitestosterone antiserum and gonadotrophin standard.

The mean of the T/E ratios was 0.98 with a 95% range of 0.31 to 1.65 and of the T/LH ratios was 26.4 nmol/IU with a 95% range of 1.1 to 51.7 nmol/IU. The ratios were plotted as frequency histograms, both sets of data giving a positive skew (*Figures 1a* and *2a*). To meet the requirements of parametric statistical analysis as described by Mortimer and Lenton[14], the data were then subjected to transforma-

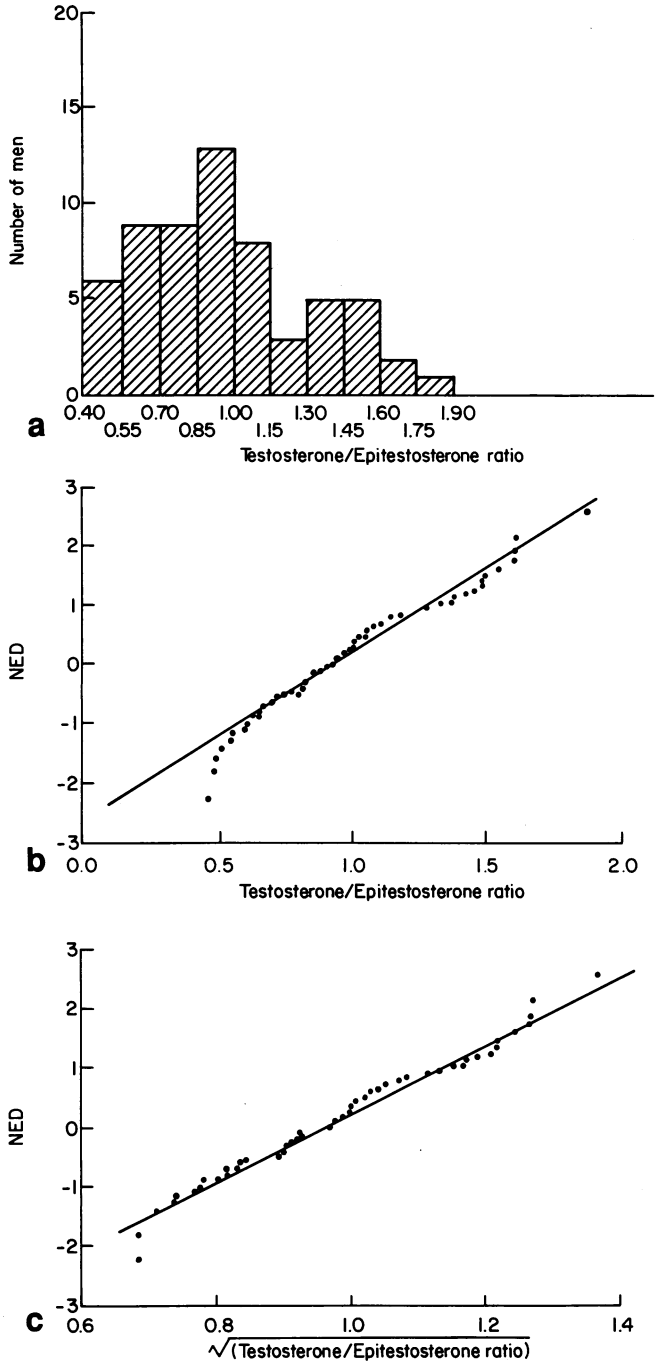

Figure 1. Histogram (a) and NED plots of untransformed (b) and transformed (c) T/E ratios (determined using commercially supplied epitestosterone antiserum)

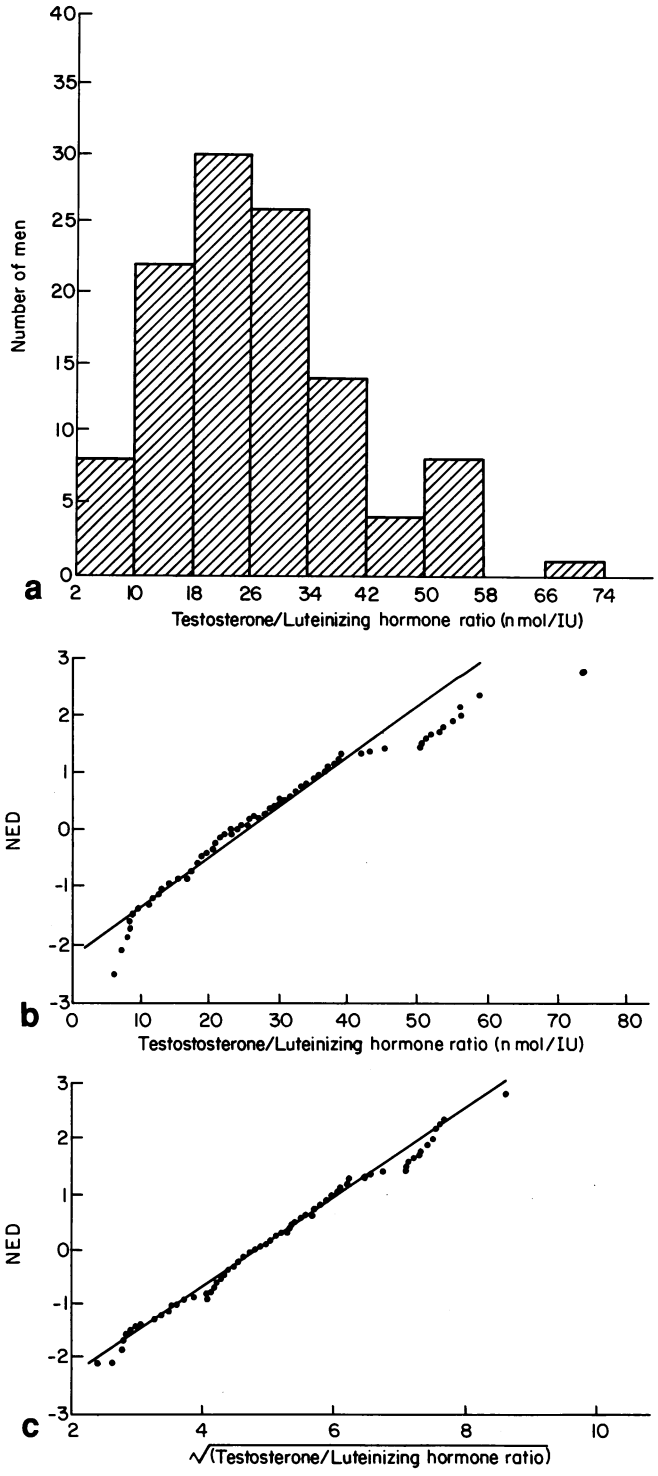

Figure 2. Histogram (a) and NED plots of untransformed (b) and transformed (c) T/LH ratios (determined using HMG reference preparation)

tions and compared using normal equivalent deviate (NED) plots. The square root transformations of the T/E and T/LH ratios (*Figures 1c* and *2c*) gave the best linear relationship when compared to the untransformed data (*Figures 1b* and *2b*) and were used for further statistical analysis. The means, as determined from the transformed data, were 0.95 for the T/E ratios (95% range of 0.41 to 1.72) and 24.9 nmol/IU for the T/LH ratios (95% range of 6.6 to 55.1 nmol/IU).

The probability of obtaining a value greater than five standard deviations (calculated using number of observations minus one) above the mean of a Gaussian distribution is 3×10^{-7}. This was interpreted with some reservation, that three in ten million normal men will have a T/E ratio more than 3.4 or a T/LH ratio more than 125 nmol/IU and these values were chosen as suitable cut-off points.

Responses to testosterone heptanoate administration and HCG stimulation

Serum

The percentage changes from basal values (day 1) of serum concentrations of testosterone, LH, FSH, oestradiol, SHBG and prolactin are illustrated in *Figure 3*.

The serum concentrations of testosterone rose between 200 and 411% for the three subjects in the first 24 hours after the testosterone heptanoate injection. The testosterone concentrations then fell progressively, so that on the day of HCG stimulation (day 15), the values were approximately half the basal concentrations. After HCG stimulation, the testosterone concentration increased in all three subjects. By day 19, four days after HCG administration, the serum testosterone values had increased by 26 to 68% above basal values, representing an approximate three fold rise from the depressed values observed on day 15. The subjects' testosterone concentrations had fallen to basal levels eight days after stimulation, and were below basal values 11 days after HCG administration.

The serum LH concentrations fell in all three subjects after testosterone administration and were still 50% below basal levels for subjects 1 and 3 on day 12. After HCG stimulation, the LH values increased dramatically in all three subjects due to the high cross-reaction of HCG with the LH antiserum. Maximal increases were observed 24 hours after stimulation, subject 1's values increasing by over 1200%.

The serum FSH concentrations also fell progressively after testosterone administration and were still suppressed below basal values for subjects 1 and 3 on day 12. The values increased up to between 20 and 76% above basal values after HCG stimulation due to a small cross-reaction of HCG with the FSH antiserum.

The serum oestradiol concentrations increased immediately after testosterone administration and rose as high again after HCG stimulation.

The changes in SHBG concentrations after testosterone administration were not immediately consistent for all three subjects, but by day 5 all three subjects' SHBG values had decreased between 11 and 21%. Thereafter, the SHBG concentrations remained suppressed throughout the experiment.

The serum prolactin concentrations increased after testosterone administration for all three subjects and increased again for subject 1 after HCG stimulation.

Urine

Changes in T/E and T/LH ratios are displayed in *Figure 4*.

The ratio of T/E showed a pronounced rise above the cut-off point in all three subjects after testosterone heptanoate injection. The ratios rose from basal values of 0.66–0.72 to maximum values between seven and eight on day 5, four days after administration. This rise represented a 10 to 11 fold increase from basal values. Two weeks after testosterone administration, the ratios had returned to approximately normal levels.

Table 2 displays the total urinary excretion in 24 hours of testosterone and epitestosterone for collections day 0 to 1, 2 to 3 and 7 to 8. Although testosterone excretion rose markedly after testosterone administration, there was a decrease in epitestosterone excretion by day 3 followed by a further fall by day 8. This decrease in epitestosterone excretion was most pronounced for subject 1 whose values fell by 65% from the basal rate of 892 nmol/24 hours to 304 nmol/24 hours by day 8.

After HCG stimulation, the T/E ratios rose marginally for each subject, the maximum value of 1.8 being observed for subject 3 on day 19, four days after stimulation. All the maximum values were less than the highest normal ratio of 1.86 observed in the medical students. The marginal increase in urinary T/E values was sharply contrasted by the three fold rise of serum testosterone after HCG stimulation.

The changes of T/LH ratios after testosterone

Table 2. Total 24 hour urinary excretion of testosterone and epitestosterone (nmol), basal values and after i.m. injection of testosterone heptanoate (Primoteston®, 250 mg) on day 1

Day of collection	*Subject 1*		*Subject 2*		*Subject 3*	
	Testosterone	*Epitestosterone*	*Testosterone*	*Epitestosterone*	*Testosterone*	*Epitestosterone*
0–1 (Basal)	559	892	727	794	281	388
2–3	1748	595	1987	636	1281	326
7–8	515	309	788	534	1365	258

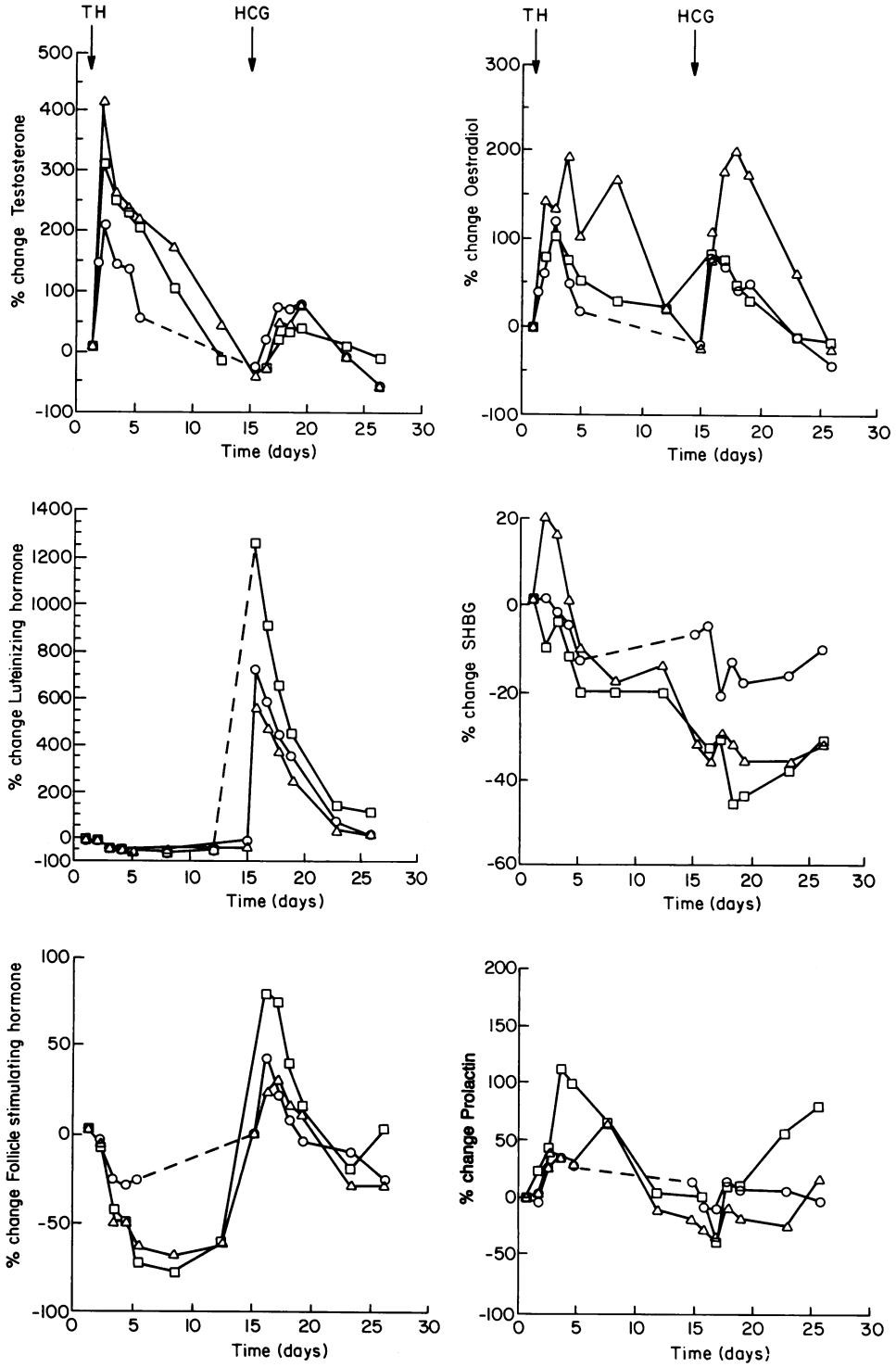

Figure 3. The percentage change from basal serum concentrations of testosterone, LH, FSH, oestradiol, SHBG and prolactin, after administration of testosterone heptanoate (TH) followed by HCG stimulation, for subjects 1(□), 2(○) and 3 (△)

administration showed a similar pattern to that observed for T/E ratios although subjects 1 and 3's maximum ratios were on day 7 in contrast to their T/E ratios which peaked on day 5. The maximum values of 315 to 448 nmol/IU for the three subjects were 10 to 20 times above their basal ratios which was a similar distortion as that shown by the T/E ratios.

After HCG stimulation, the T/LH ratios fell for one to two days below the ratio of 6.6 (the lower value of the 95% normal range of square root transformed data). The urinary T/LH ratios fell to low values due to the cross-reaction of HCG with the LH antiserum.

As seen from *Table 3*, HCG was detected in urine using the Alpha Laboratories kit up to day 21 for all three subjects, six days after stimulation. By day 23 there was little or no HCG observed. The qualitative results of the kit corresponded well with the quantitative results of the radioimmunoassay for HCG detection.

Table 3. Comparison of urinary HCG results between enzyme linked immunoassay kit and Charing Cross Hospital specific radioimmunoassay after injection of HCG

Day	*Subject*					
	1		*2*		*3*	
	Kit	*C.X.*	*Kit*	*C.X.*	*Kit*	*C.X.*
14	–	n.p.	–	n.p.	–	n.p.
15	–	<2	–	<2	–	<2
HCG 5000 units	---------->		---------->		---------->	
16	++++	298	++++	181	++++	113
17	++	68	++	76	++	92
18	+++	209	+++	129	++	98
19	++	58	++	74	++	94
21	++	42	n.p.	n.p.	++	52
23	–	2	+	8	+	23
26	–	14	–	15	n.p.	n.p.

Kit: – = negative; + = 25 IU/l; n.p. = not performed
Charing Cross assay (C.X.): Definitive detection limit = 20 IU/l

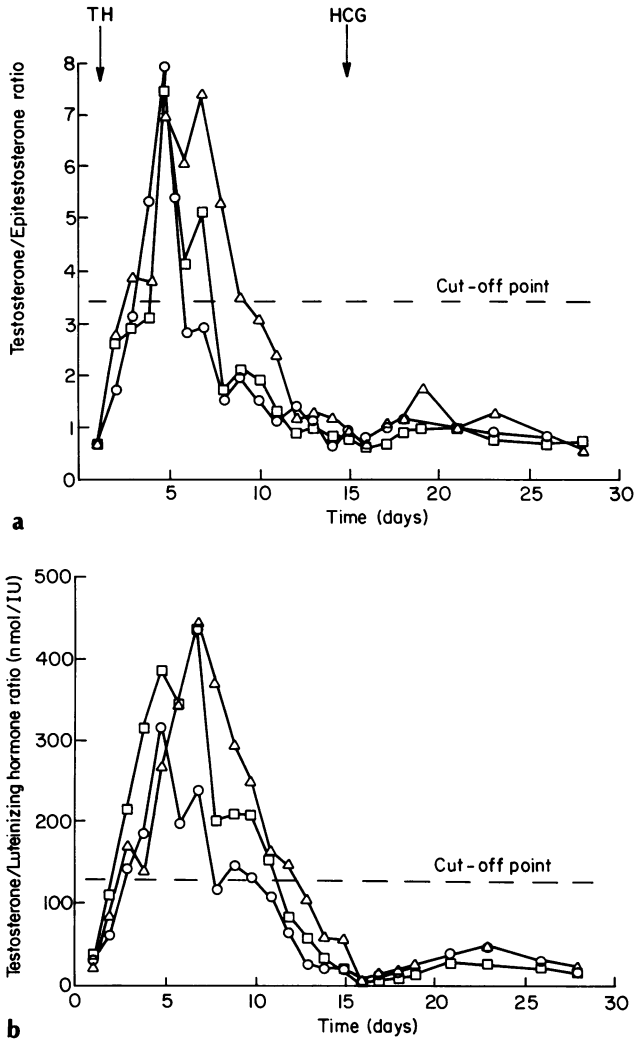

Figure 4. The effects of the administration of testosterone heptanoate (TH) followed by HCG stimulation on (a) urinary T/E and (b) T/LH ratios for subjects 1(□), 2(○) and 3(△)

Normal ranges of T/E and T/LH ratios in male urine using our own laboratory produced epitestosterone antiserum and 1st IRP Pituitary Gonadotrophin Standard were assessed.

A new T/E normal range was determined because our own laboratory produced epitestosterone antiserum gave a lower summated cross-reaction with androsterone and aetiocholanolone (0.14% at 30% displacement) compared to the commercially supplied antiserum (0.91% at 30% displacement). A new T/LH normal range was also determined because of the replacement with the 1st IRP Pituitary Gonadotrophin Standard.

The frequency histogram plots of the T/E and T/LH ratios together with the untransformed and transformed NED plots are shown in *Figures 5* and *6*. The

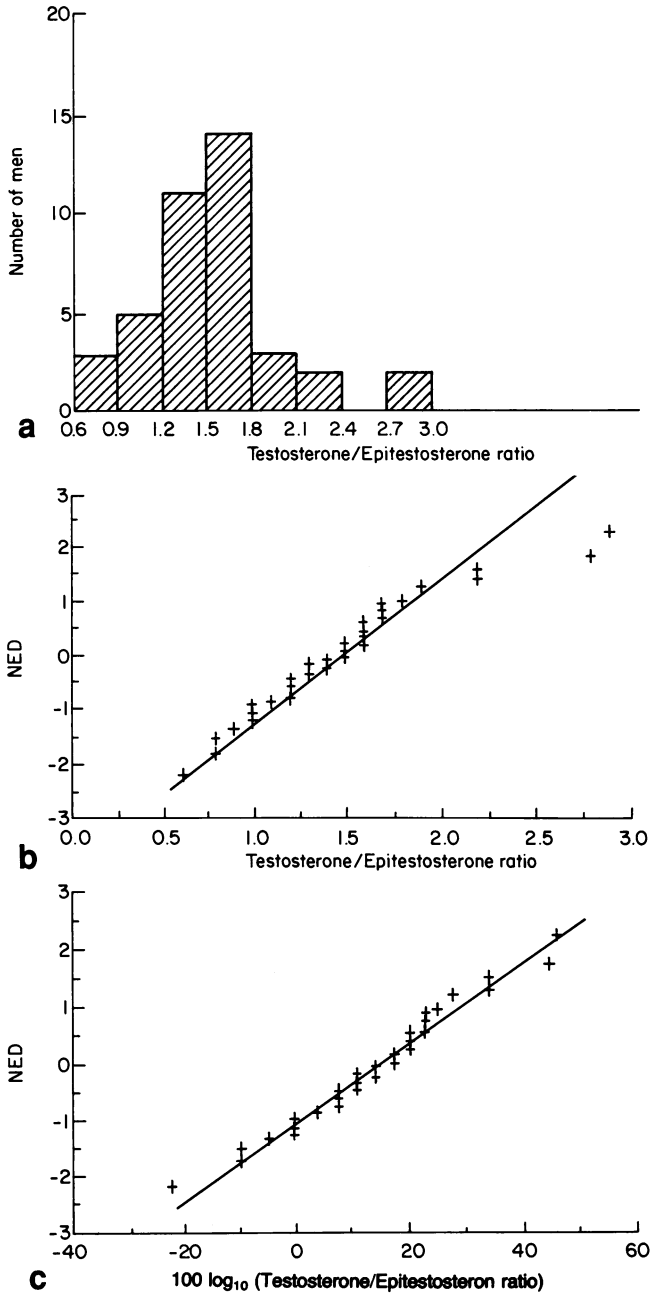

Figure 5. Histogram (a) and NED plots of untransformed (b) and transformed (c) T/E ratios (determined using own laboratory epitestosterone antiserum). In (a) $\bar{x}$ = 1.48

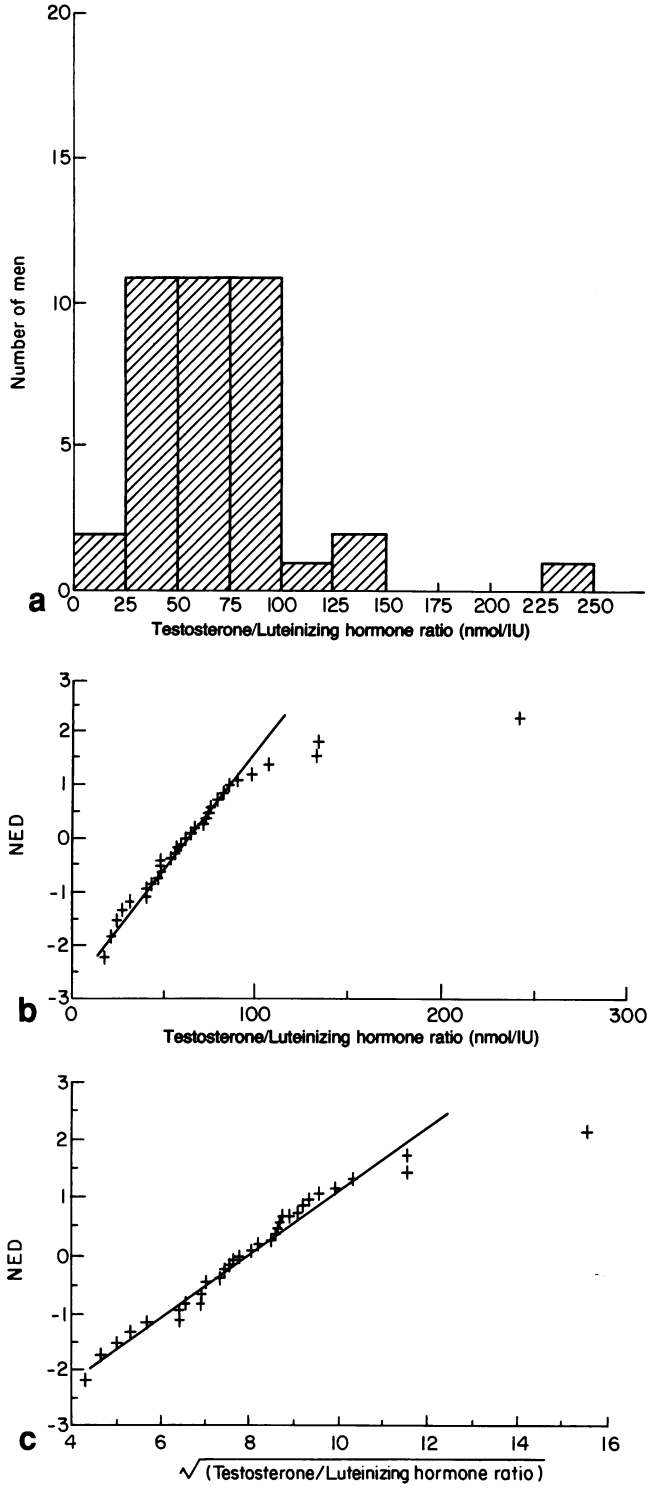

Figure 6. Histogram (a) and NED plots of untransformed (b) and transformed (c) T/LH ratios (determined using 1st IRP pituitary LH standard). In (a) $\bar{x}$ = 6.95 nmol/IU

mean of the untransformed data was 1.48 for the T/E ratios with a 95% range of 0.55 to 2.41 and for the T/LH ratios was 69.5 nmol/IU with a 95% range of −7.3 to 146.3 nmol/IU. The logarithmic transformations of the T/E ratios and the square root transformations of the T/LH ratios gave the best approximations to a Gaussian distribution (T/E: $\bar{x}$ = 1.44, 95% range = 0.68 to 2.48; T/LH: $\bar{x}$ = 65.3, 95% range = 16.2 to 147.3). Five SD above the means of the transformed

Table 4. The T/E and T/LH ratios before and after vigorous exercise

Karate subject	*T/E ratios* Before exercise	*T/E ratios* After exercise	*T/LH ratios* (nmol/IU) Before exercise	*T/LH ratios* (nmol/IU) After exercise
1	1.69	2.01	36.2	32.9
2	1.52	1.42	75.5	32.9
3	2.22	2.07	52.0	45.3
4	2.24	2.18	61.9	70.2
5	1.17	1.75	57.7	64.0
6	1.14	1.59	85.7	64.6
7	2.78	3.19	51.3	38.1
8	1.62	1.59	50.6	47.9
9	1.16	1.53	51.9	53.6

data gave cut-off points of T/E = 7.0 and T/LH = 340 nmol/IU.

The effect of vigorous exercise on urinary T/E and T/LH ratios was also demonstrated. The urinary T/E and T/LH ratios before and after exercise are displayed in *Table 4*. After conversion of the T/E ratios to their logarithms and the T/LH ratios to their square roots, the data were subjected to a paired Student t-test. The T/E data gave a value of $0.2>P>0.1$ and the T/LH data gave a value of $0.3>P>0.2$. In both cases $P>0.05$ (5%) so the null hypothesis was not disproven, i.e. there was no significant change in the T/E or T/LH ratios after exercise.

The T/E ratios of subject 7 increased from 2.78 before exercise to 3.19 after exercise, which was just above the top value of the range of the data. However, the value of 3.19 represented only 2 to 3 standard deviations from the mean of the transformed data.

Figure 7 shows the effect of the administration of the mixture of testosterone and epitestosterone heptanoates on the urinary T/E and T/LH ratios as measured by radioimmunoassay. Only the T/LH ratios rose to well above the cut-off point, the T/E

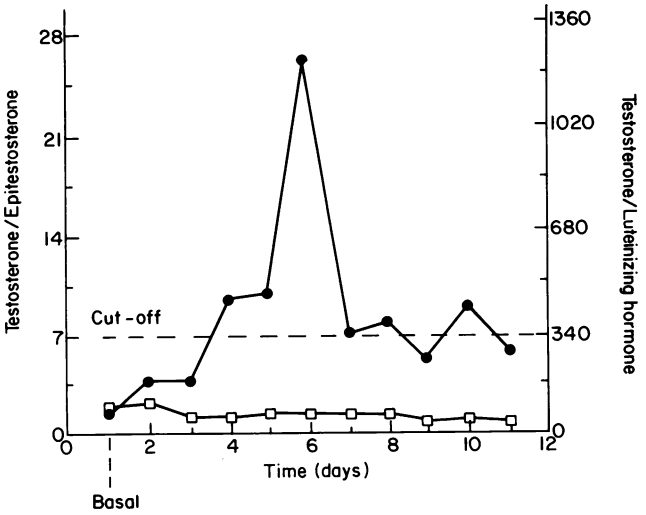

Figure 7. T/E (□) and T/LH (●) ratios following the administration of the combined testosterone-epitestosterone heptanoate preparation to a healthy adult male on day 1

ratios showing no significant change. This was in marked contrast to the ten fold increase in the T/E ratio of the same subject following the administration of an identical dose of testosterone heptanoate alone (*Figure 4* – subject 2).

Using GLC-MS, the T/E ratios determined gave normal values (range 0.6 to 1.2) which agreed with the results obtained by radioimmunoassay (range 0.8 to 2.1) as displayed in *Table 5*.

Discussion

Testosterone heptanoate administration increased the serum total testosterone concentration to supraphysiological values within the first 24 hours for all three subjects. The serum testosterone remained above basal values for over ten days, which is typical for this formulation.

The rapid rise in serum testosterone was reflected by a sharp decrease in the serum gonadotrophins within the first 48 hours of the experiment. The magnitude and duration of the depression of both gonadotrophins were approximately equal, a finding which was in agreement with Cunningham *et al.*[15]. This indicates that the negative feedback mechanism incurred by supraphysiological doses of testosterone appears to apply to both gonadotrophins.

After HCG stimulation, the serum LH and FSH values increased due to HCG cross-reacting with the respective antisera.

The high concentrations of serum testosterone after exogenous testosterone administration was accompanied by marked increases in blood oestradiol concentrations due to the peripheral conversion of androgens to oestrogens. About 75% of the total oestradiol production in normal men can be accounted for by the conversion from androstenedione and especially testosterone in extraglandular sites[16,17].

The oestradiol concentrations rose as high again after HCG stimulation, although the serum testosterone increases were less pronounced than those after testosterone heptanoate administration. The stimulatory effect of HCG on human testicular steroidogenesis is well documented, leading to an increase in blood concentrations of both androgens and oestrogens. The large rise in oestradiol could be attributed mainly to an increase in the testicular secretion of this steroid, with a small contribution from the peripheral conversion of the rising plasma testosterone concentration. This was because there was a much smaller increase in testosterone after HCG stimulation than that after testosterone heptanoate administration but approximately equal increases in oestradiol in both cases. The testicular response to HCG, whereby the oestradiol maximum preceded the testosterone maximum, is in accordance with findings in other studies[18–20]. Saez and Forest showed that there was no direct correlation between the plasma levels of HCG and testosterone in men[21]. The hypothesis favoured by many authors is that oestradiol plays an intratesticular role in androgen production.

The oestradiol concentration of subject 3 was still very high four days after HCG stimulation, on the day of maximum testosterone concentration. This subject may have had a higher peripheral conversion of testosterone to oestradiol than the other two subjects. This suggestion is supported by the much higher concentration of oestradiol present seven days after testosterone heptanoate administration.

Serum SHBG fell after testosterone administration which was in accordance with other studies[22]. Ruokonen *et al.* showed that power athletes who self-administered very large doses of testosterone and synthetic androgenic-anabolic steroids had depressed concentrations of serum SHBG 16 weeks after drug withdrawal[23].

The increase in serum prolactin after depot testosterone administration was in agreement with the study by Ruiz *et al.*[24]. Prolactin stimulates testicular function within the physiological range but is inhibitory in excess[25]. The secretion of this hormone is under a complex dual control, involving inhibitory and stimulatory factors via neuroendocrine and possible autocrine and paracrine mechanisms[26].

There is evidence to support a paracrine relationship between lactotrophs and gonadotrophs in rat pituitary cell cultures and that gonadotrophin releasing factor stimulates prolactin release in humans[27]. Paradoxically, although the supraphysiological dose of testosterone in this experiment suppressed LH release, it stimulated prolactin release.

The square root transformed data of the urinary T/E and T/LH ratios (using urinary human menopausal gonadotrophin standard and commercial epitestosterone antiserum) provided the best normalization of both distributions, as was shown by the parity between the mean and median values. Overall, there was little difference between the means and 95% ranges of the untransformed and transformed data for the T/E or T/LH ratios. However, the normalization of the data justifies, with some reservation due to the small number of samples analysed, the quoting of probabilities for values above the mean. We postulate

Table 5. Urinary T/E ratios obtained by immunoassay and GLC-MS, basal values and values following i.m. injection of the heptanoate esters of testosterone and epitestosterone (28:1 w/w) to one subject on day 1

Day	*T/E ratios*	
	RIA	*GLC-MS*
1 (Basal)	1.7	N.P.*
2	2.1	0.9
3	1.2	0.8
4	1.2	1.0
5	1.3	1.2
6	1.4	1.0
7	1.4	0.9
8	1.3	0.8
9	0.8	0.8
10	1.0	0.7
11	0.8	0.7

*N.P. = not performed

with additional data that an athlete with both urinary ratios 5SD from the mean could be considered positive for androgen administration.

A larger sample number would have been desirable for statistical analysis but the use of two separate urinary indices, combined with a generous cut-off point for positive samples, would ensure a viable method for assessing testosterone administration with a higher degree of confidence. Should the IOC accredited laboratories decide to use more than one ratio they would have to agree on standard methods and then a group of laboratories could carry out the measurements on the several hundred cases needed to define the normal population.

The viability of using both urinary T/E and T/LH ratios was confirmed by the large increase in the ratios after testosterone heptanoate administration for all three subjects. The urinary ratios increased proportionately more than the serum testosterone because of suppression of LH and epitestosterone production. It follows that repeated injections of testosterone heptanoate at weekly intervals would have a cumulative effect with more complete gonadotrophin suppression and further increases in the T/E and T/LH ratios. The fall in urinary excretion of epitestosterone was indirect evidence that this hormone is secreted by the testes. This was important as it indicates that any possible peripheral conversion of large concentrations of testosterone to epitestosterone is outweighed by the suppression of epitestosterone production. In addition, HCG stimulation did not cause as large a rise in the urinary T/E ratios because HCG also stimulates epitestosterone production[28].

By contrast, the T/LH ratios fell significantly shortly after HCG stimulation due to the cross-reaction of HCG with the LH antiserum. This suggested that samples with T/LH ratios below or on the lower end of the normal range may be an indication of HCG doping. Indeed, this was demonstrated shortly after this study when routine testing of sports samples in the laboratory revealed two samples with low T/LH ratios. These two urines were found to have very high concentrations of HCG as a result of which a large number of male urines from sport were screened for HCG. The degree of HCG abuse found in this study led to the inclusion of HCG in the list of banned substances by the International Olympic Committee. Of course, using an LH antiserum that does not cross-react with HCG would give T/LH values above the normal range after HCG stimulation[29].

The modified pregnancy testing kit performed well for the detection of HCG in male urine. The kit results combined with the HCG radioimmunoassay values showed that HCG could be detected throughout the period of elevated serum testosterone due to HCG stimulation.

A new urinary T/E normal range using the epitestosterone antiserum prepared in this study was evaluated. This was essential as the cross-reactions of androsterone and aetiocholanolone with our own laboratory produced epitestosterone antiserum were much smaller than with the commercially supplied antiserum. As the concentrations of these two urinary 17-oxo-steroids are considerably greater than that of epitestosterone, they could significantly interfere in the assay using the commercially supplied antiserum. This fact was demonstrated by the disparity between the determined T/E normal range using the commercially supplied antiserum and the home produced antiserum. The new T/E cut-off point of 7:1 was in close proximity to the IOC recommended cut-off point of 6:1 when determined by GLC-MS.

When the supply of the urinary gonadotrophin standard was withdrawn, human pituitary LH standard was recommended for urinary LH radioimmunoassays. This pituitary LH standard was considerably purer and thus necessitated the determination of a new normal T/LH range.

The switching of the commercially supplied epitestosterone antiserum to our own laboratory produced epitestosterone antiserum and the change to a much purer LH standard illustrate that the determination of cut-off points in drug control must depend on the methods used to quantitate the analytes. It is not suggested that immunoassay would be adopted by the IOC accredited laboratories to quantitate testosterone and epitestosterone because these measurements are more easily obtained by GLC-MS. Having determined the frequency distribution of the T/E and T/LH ratios with our own produced epitestosterone antiserum and pituitary LH standard (albeit on a small sample number), it seemed of value to demonstrate the statistical significance of the data and the effects of exercise and administration of testosterone-epitestosterone esters on the ratios.

Serum testosterone concentrations may rise during long intensive exercise[30], probably due to a decreased hepatic clearance. Immediately after exercise, concentrations of serum testosterone and in some cases LH as well, have been shown to decrease. This may be because of a negative feedback effect of the rising testosterone or oestradiol during exercise[31]. Nevertheless, the urinary T/E or T/LH ratios were not significantly changed by intense exercise in the nine karate subjects. Thus, any transient changes in serum testosterone, oestradiol or LH during or immediately after, long intensive exercise are of insufficient magnitude or duration to affect the urinary T/E or T/LH ratios.

The administration of the combined testosterone-epitesterone esters in the ratio of 28:1 demonstrated that it is possible to evade detection if this is dependent only on the T/E ratio. Although epitestosterone heptanoate is not a normal pharmaceutical product, it is very easy to prepare and in view of the statements by Dr B.H. Wooley on designer drugs (1st IIAF Symposium on Doping in Sport, Florence 1987) it would be very complacent to assume that it is unavailable to athletes. With the high incidence of testosterone positives in recent years and the increasing sophistication of drug abuse in sport, it would seem prudent to adopt the T/LH test as a primary screen.

Acknowledgements

To the Sports Council for supporting this study.

Thanks are also due to Dr Hugh Mitchell and staff of the SAS Laboratories at Charing Cross Hospital for the assaying of urinary HCG; to Paul Levy of the Drug Control and Teaching Centre for measuring the urinary T/E ratios by GLC-MS; finally, to the nursing staff of Louise Ward, St Thomas' Hospital, for carrying out the injections.

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