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Screening for testosterone abuse in male athletes using the measurement of urinary LH, a revision of the paradigm

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The primary screening method for the detection of doping by athletes using synthetic versions of endogenous steroids such as testosterone relies on measurement of the ratio of testosterone (T) to epitestosterone (E) in urine. In 2005 the World Anti-Doping Agency (WADA) lowered the T/E value at which samples undergo further investigation from six to four. This has resulted in a large increase in the number of athletes with naturally elevated T/E ratios undergoing investigation without a corresponding increase in the number of proven doping offences involving testosterone.

Our objective was to develop a new simple screening protocol that can, with high probability, not only distinguish athletes whose natural T/E values exceed four from those whose T/E values have been elevated by testosterone doping but also detect those athletes with naturally low T/E values that do not exceed four despite being administered testosterone.

Testosterone (250 mg Sustanon) was administered weekly to a group of 47 young adult males for five weeks in a double-blind placebo controlled study and urine samples collected. The samples were analysed for steroid concentrations using GC/MS and for luteinizing hormone (LH) by immunoassay.

The elevation of T/E that occurred in all subjects was accompanied by a significant reduction in urinary LH concentrations to levels that are rare in normal subjects.

The appropriate measurement of urinary LH, with the measurement of T/E values, can markedly improve the efficiency of detection of doping with testosterone by male athletes, particularly those who have low natural T/E ratios.

Keywords: anabolic steroids; luteinizing hormone; sports medicine; T/E ratio

Introduction

The abuse of testosterone and other synthetic endogenous anabolic steroids continues to be a major problem in sport. The detection and confirmation of the abuse of exogenous anabolic steroids is relatively straightforward because the metabolites are not naturally present in urine. However with synthetic forms of 'natural' anabolic steroids, such as testosterone, the problem is much more difficult as the compound and its metabolites are endogenous. Thus methods have been sought to detect such doping by the use of the ratio of amount of testosterone present compared to that of another naturally occurring steroid, epitestosterone.[1]

Originally, population data showed that this ratio is stable in each individual and values above six were not found. The mean value for a normal population was approximately one with a positively skewed distribution.[2,3] If subjects are given testosterone then their T/E ratio will rise markedly. When the concept of using the T/E ratio was introduced in 1982 to detect doping with testosterone the action level was set at six by the Medical Commission of the International Olympic Committee (IOC). The introduction of testing on a large scale by the then IOC-accredited laboratories found that values of six and above did occur naturally in some subjects, mainly due to low epitestosterone levels.[4] Longitudinal studies to compare the variation in T/E ratio over time for an individual were introduced to help decide doping cases and to eliminate those cases where the T/E ratio was naturally elevated due to physiological causes.

In 1997, Shackleton *et al*.[5] described a practical approach using carbon isotope ratio mass spectrometry (CIRMS) as a means of confirming doping with testosterone. Synthetic testosterone has been found to be carbon 13 depleted compared to endogenous testosterone and thus by measuring the CIR of testosterone metabolites it was possible to detect testosterone abuse. However CIRMS is not routinely used for screening samples because it is a lengthy and expensive process. Measurement of T/E is still the primary means of screening for possible doping with testosterone and related synthetic endogenous steroids.

There are, however, problems in relying on T/E measurement alone to detect such doping. Whilst most of the population will produce T/E values above six when given testosterone, there is an ethnic group with natural T/E values well below the population

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mean, which will not produce T/E values above six despite being given testosterone.[6] In 2005 the WADA Prohibited List reduced the T/E level at which further investigation was required from six to four. This had the unfortunate effect of making the international federations and anti-doping organizations carry out many more investigations, the vast majority of which are unnecessary because up to 2% of normal subjects have T/E levels above four.[7] The result has been a major increase in follow-up testing and the use of CIRMS both of which have added significantly to the cost of drug testing with very little impact on the number of confirmed cases of testosterone doping.[8,9] The reason for this failure is that those who were previously not detected at T/E above six are unlikely to be detected with the lowered value of four. Until individual reference ranges are available it is not possible to detect efficiently low-mode T excretors who dope with testosterone using T/E measurements alone.[10,11] An effective and inexpensive screening test is needed to both detect the low-mode excretors who, when given testosterone, do not produce T/E values above four and eliminate those subjects whose normal T/E value is above four. The results of a recent study on the administration of testosterone to a group of male recreational athletes have shown that the measurement of luteinizing hormone (LH) in the urine of males is a very useful tool for the detection of testosterone doping. This is to be expected given the relationship between testosterone and LH in the hypothalamic pituitary-testicular axis. Exogenous testosterone is expected to suppress natural production of testosterone resulting in lower circulating concentrations of LH.

The use of LH for detecting testosterone doping was suggested by Brooks *et al*. in 1979.[12] This was done by measuring the urinary testosterone to LH ratio (T/LH). Further studies confirmed the usefulness of measuring the T/LH ratio[13,14,15] and it was reported that the T/LH ratio was of more use in detecting testosterone doping than the T/E ratio.[16] However, such measurements have not been used routinely on all samples as a screen for testosterone doping. Measurements of LH are often only made for confirming samples with elevated T/E values and then the T/LH ratio is used. Our proposal is to measure LH in all urine samples collected from males and use the LH concentration, rather than the T/LH ratio, together with the T/E ratio as the primary means of screening for doping with testosterone. Suspicious samples would then be further tested using confirmatory techniques such as CIRMS to allow adverse analytical findings to be reported.

Unfortunately the methodology is not directly applicable to females because oral contraceptive therapy also suppresses LH secretion.

Materials and Methods

The urine samples were collected as part of a study conducted with the aim of detecting growth hormone (GH) abuse markers with and without the influence of testosterone. The study was registered with the Australian New Zealand Clinical Trials Registry (ACTRN012605000508673, www.anzctr.org.au) and the full details of the protocol used have been published.[17] A summary of the randomized double-blind placebo-controlled protocol used is shown in Figure 1. The subjects were young adult males with 16 in each of three groups: testosterone plus GH, testosterone plus placebo GH, and double placebo. Their mean age was 29 years. The GH (Somatropin, and matched GH placebo were provided by Novo Nordisk (Bagsvaerd, Denmark)).

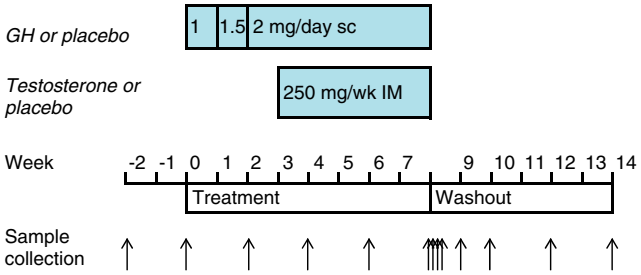

Figure 1. Administration study protocol.

The GH administration began in week 1 at 1 mg/day and was given from weeks 3 to 8 at 2 mg/day. The 250 mg mixture of testosterone esters containing 176 mg of testosterone (Sustanon®, Organon, Oss, Holland), or placebo saline, was administered once per week intramuscularly for five weeks, from the end of week 3 until the end of week 7. Single-pass urine samples were collected two weeks before the study began and at weeks 0, 2, 4, 6, 8, 9, 10, 12 and 14. Three additional samples were collected in week 8, one day apart, from each subject making a total of 13 possible samples from each subject. The samples were stored at −20 °C until analysed (a period of approximately two years). Serum samples were also collected and stored at −80 °C until analysed (a period of approximately one year).

The urinary steroid concentrations were measured using gas chromatography mass spectrometry after enzymatic hydrolysis using standard procedures for sports drug testing.[18] The GC/MS measurements included T/E ratios and concentrations of testosterone, epitestosterone, androsterone, etiocholanolone, DHEA, and the 5α and 5β-androstane-3α, 17β-diols. The specific gravity (SG) of the urine samples was measured using a refractometer (UG-1, Atago Co., Japan). The LH concentrations were measured using a DPC Immulite kit from Siemens Australia (LKLH1).

The urine samples were equilibrated to room temperature, shaken to mix and aliquoted without filtration. The Immulite LH assay was developed and validated for measuring LH in serum and the urine aliquots were treated in the same manner as serum. The reproducibility of the assay was determined by carrying out seven replicate measurements on 10 different urine samples with measured LH values of between 0.8 and 41 IU/L. The median CV was 4.2% with the lowest being 2.4% and the highest 15.2%. The linearity of the measurement was established by serially diluting a urine sample with a measured LH of 18.0 IU/L with another urine having a suppressed LH of 0.6 IU/L and making replicate measurements (minimum four) at each dilution. The slope of the line of best fit from measured versus calculated LH was 0.95 with $R^2 = 0.99$. The recovery was calculated by spiking a urine sample with a measured LH concentration of 17.4 IU/L with an additional 17 IU/L of WHO pituitary LH (NIBSC 80/552). The new measured value was 35.7 IU/L giving a recovery of 108%. The limit of detection in urine was estimated to be 0.4 IU/L. The effect of storage at – 20 °C on urinary LH concentrations was evaluated by storing 46 urine samples (mean 9.0 IU/L, range 0.4 to 47 IU/L) for two years and then reanalysing them. The relationship between the values was:

$$LH_{2\ years} = 1.02\ LH_{initial}.\ \text{The } R^2 \text{ was } 0.89.$$

The LH values were adjusted for SG using the formula below, which has been used widely to adjust urinary concentrations for

SG variation:[19]

$$LH_{adjusted} = LH \times ((1.020 - 1)/SG - 1))$$

Results

In all, some 390 urine samples were collected from 31 male subjects who had been given five 250 mg doses of testosterone ester starting at the end of week 3 and finishing at the end of week 7. Sixteen of the subjects were also being administered GH at a dose of 2 mg/day during this period. A second group of 16 male subjects was given placebo injections. The results from all 31 subjects given testosterone have been pooled as there was no significant difference detected between the two administration groups in any of the measured results obtained. This decision was supported by the observation that the data from a fourth group of male subjects who were only administered growth hormone displayed no changes in their steroid profile or LH results, which could be correlated with the administration of growth hormone (data not shown).

The three urine samples collected from each subject prior to their first injection of testosterone were used to establish the normal steroid profiles for the subjects. As expected from previous studies,[20,21] the epitestosterone concentration fell and the testosterone concentration rose after testosterone administration, with the overall effect being a large increase of the T/E ratio for all subjects. Figure 2 shows the mean initial T/E value (labelled week 0) and the values at weeks 4, 6, 8, 9, 10, 12 and 14 weeks for all the subjects. For all subjects the T/E values rose markedly from their initial values by the week 4 collection which was a few days after the first injection at the end of week 3. The maximum values were reached in weeks 6 and 8 and by week 9 which was some 10 days after the last injection the values had begun to fall. This fall continued and the T/E values for most subjects had returned to values within their normal range by week 14, which was six weeks after the last injection of testosterone.

The results obtained from measuring the LH concentration of the urine samples after specific gravity adjustment are shown in Figure 3. The results obtained before and after the treatment period are similar to those found for the placebo group whilst the samples collected from weeks 4 to 10 are much lower. It is clear that the administration of testosterone has had a marked effect on the concentration of excreted LH. In order to ascertain whether the LH measurements in urine were a true reflection of the LH response to the testosterone administration the LH was measured in serum at a smaller number of time points. The results are shown in Figure 4, where it can be seen that the serum LH was suppressed during the treatment period as it was for the urinary LH measurements.

The primary reason for choosing to use SG-adjusted LH values was to overcome the problem of low LH concentrations due to dilute urine samples. If a cutoff of 2 IU/L was used then more than 10% of samples in the placebo group fell into this category whereas only 2% did so after SG adjustment. Figure 5 shows scatter plots of T/LH and SG adjusted LH over time and mean values at each time point for the entire group treated with testosterone. All subjects given testosterone displayed a significant reduction in urinary LH during the treatment period. The diminution was evident at the week 4 collection but the minimum values were not reached until the week 6 and week 8 collections. The values stayed low until week 9 for all subjects but by week 10 most subjects

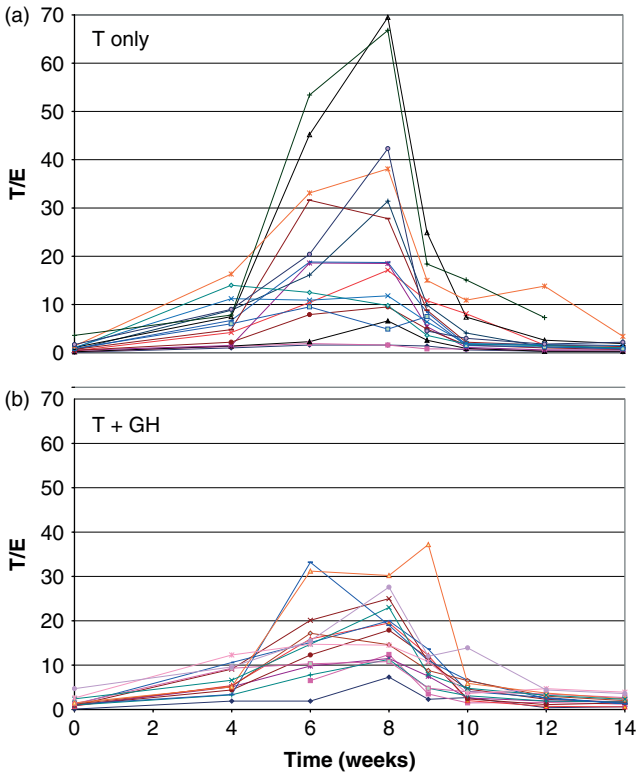

Figure 2. T/E values for all subjects over the administration trial with (a) showing the values obtained for those subjects given testosterone only and (b) showing the values for those subjects given both testosterone and growth hormones.

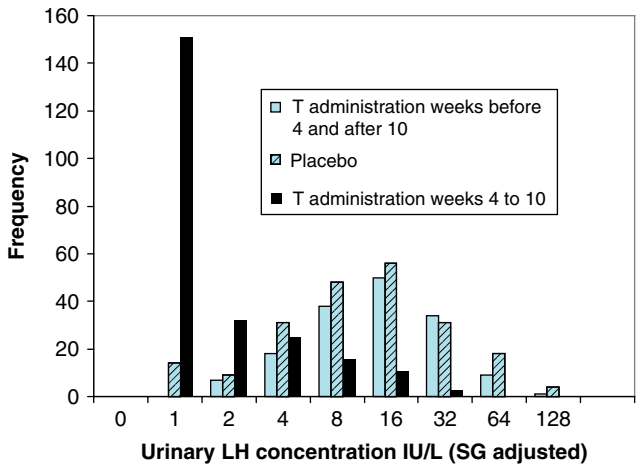

Figure 3. LH concentrations adjusted for specific gravity in both placebo and testosterone treated groups. The adjusted LH is calculated as LHadjusted = LH × ((1.020 − 1)/SG − 1)).

were returning to normal levels and by week 12 most subjects no longer had suppressed LH values. In the placebo group, adjusted concentrations of LH of 2 IU/L or below occur with a frequency of approximately 2% whereas in the treated group between weeks 4 and 10 almost 80% of the samples were below 2 IU/L. In the treatment group over the period from six to nine weeks, 168 of the 180 samples (93%) had adjusted LH values of 2 IU/L or below whilst in the placebo group over the same time, two of the 90 samples (2%) were below two. It is interesting to note that the

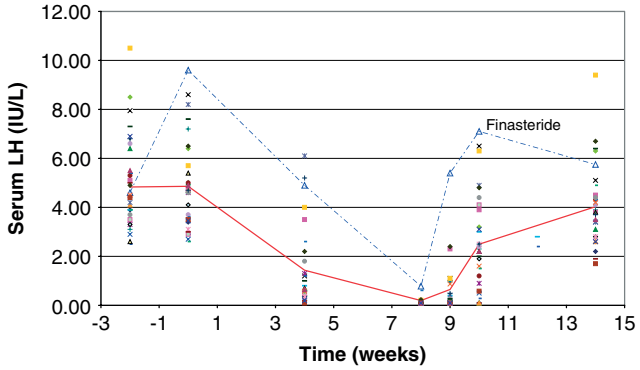

Figure 4. Scatter plot of serum LH during testosterone administration study. The solid line shows the mean values and the dotted line marks the high values found for the subject taking finasteride.

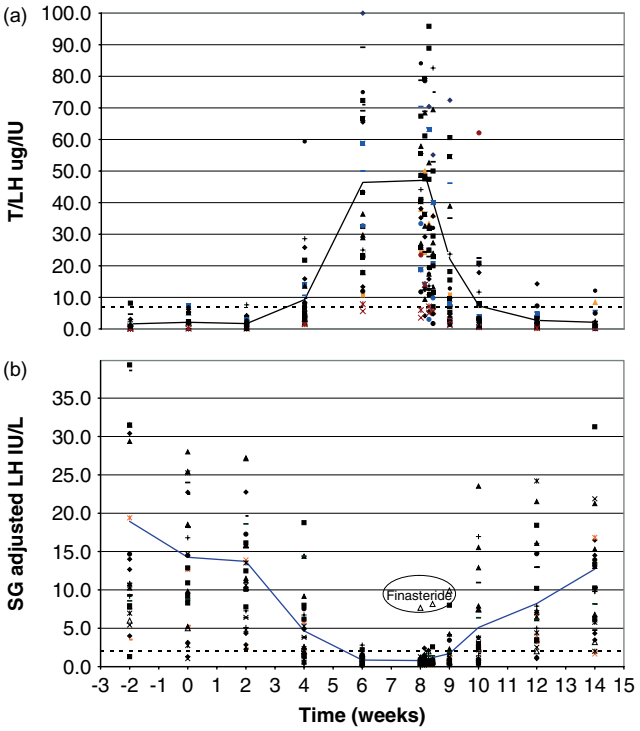

Figure 5. Scatter plots of urinary T/LH ratios (a) and SG adjusted LH concentrations (b) during testosterone administration study for 31 subjects. The vertical axes have been truncated at 100 and 40 respectively to more clearly show the effects. There were 13 samples with T/LH > 100 in the treatment period and 6 samples with LH > 40 pre treatment. The solid lines show the mean values and the dotted lines are suggested cut-off values. The LH values for the subject taking finasteride have been circled.

only samples with relatively high LH values from any of the three collections made in week 8 were from a single subject who was found to have also been taking finasteride.

Discussion

The 2009 WADA Prohibited List states that subjects with T/E values above four are to be the subject of further investigation.[22] Using this criterion, the four subjects with initial T/E values of 0.2 or below would escape detection most of the time with only two values in the collections from weeks 4 to 10 being above four (6.6 and 7.3 at week 8). It is likely that these subjects have the UDP-Glucuronosyl Transferase 2B17 (UGT2B17) polymorphism referred to by Jakobsson *et al*.[23] The WADA guideline for the reporting and management of elevated T/E ratios states that the range of values normally found in males has a 30% variation from the mean of an individual's results.[24] This effectively means that a single sample has to have a value more than twice that of the mean of three normal samples for it to be regarded as suspicious.

Using this second criterion and comparing the T/E values to the initial values, the results in Table 1 are obtained. It can be seen that the T/E > 4 criterion is insensitive for detecting administration beyond week 9 and inefficient for those subjects with low initial T/E values. Lowering the T/E threshold from six to four had no effect on the detection efficiency in the subjects with low initial T/E values. The T/E value greater than twice the initial value criterion is much more effective giving 172 suspect results from a total of 215 samples (80%) whereas the T/E > 4 criterion only detected 119 suspect samples (55%) but implementation of such detection requires knowledge of the individual's reference range.

When a sample arrives at a WADA laboratory for testing it is completely de-identified and the laboratory does not know the normal T/E ratio of the subject providing the sample. If the athlete has been tested previously the longitudinal profile can be determined by contacting the appropriate National Anti-Doping Organization or International Sporting Federation but this is rarely achievable within the time frame of routine testing, particularly at a major event where turnaround times of less than two days are required. It is apparent from our results that the use of T/E values greater than four miss approximately one-third of all suspect samples and will usually not detect samples from those subjects with low natural T/E values who may have the deletion polymorphism in the gene coding for UGT2B17.[25] There is also the problem of dealing with false presumptive positives which are samples from subjects who have natural T/E values above four.

The measurement of T/LH has been used as a confirmation of doping for those with elevated T/E values[26] and reduction of LH with the administration of testosterone does lead to an increase in the T/LH for all our subjects (Figure 5a). The inclusion of the testosterone concentration as the numerator would be expected to have the benefit of automatically adjusting for specific gravity effects, as both testosterone and LH concentrations should be similarly affected by dilution. The testosterone concentration is also higher during the administration period, enhancing the detection efficiency. The increase in T/LH from week 0 to week 8 is almost twice that found for the decrease in LH. Despite this it is apparent, by comparing Figure 5a and Figure 5b, that there are many more samples in the week 8 and week 9 collections that do not have elevated T/LH values compared to the number of samples at the same times that do not have suppressed LH values. Most of the samples that do not have elevated T/LH values in the treatment period come from the subjects with low initial T/E values. This is because their testosterone concentrations remain very low and the T/LH ratios during the administration period are within the range for normal subjects with higher T/E values. However these same subjects all have suppressed LH values in the treatment period with only one sample being above 2 IU/L in the week 6 to week 10 collections. Evaluation of the data shows that SG-adjusted LH is more effective than the T/LH ratio in detecting testosterone

Table 1. Effectiveness of detection criteria for testosterone doping

	Criterion T/E > 4							Criterion T/E > 2 × initial value						
Initial T/E	W 4	W 6	W 8	W 9	W 10	W 12	W 14	W 4	W 6	W 8	W 9	W 10	W 12	W 14
0.1			X					X	X	X	X	X	X	X
0.1								X	X	X	X	X	X	
0.2								X	X	X	X	X	X	X
0.2			X					X	X	X	X	X		
0.4	X	X	X	X	X			X	X	X	X	X	X	X
0.5		X	X	X				X	X	X	X	X		
0.5		X	X	X				X	X	X	X	X	X	
0.6	X	X	X	X	X			X	X	X	X	X		
0.7	X	X	X	X				X	X	X	X	X	X	
0.7	ns	X	X					ns	X	X	X	X	X	
0.7	X	X	X	X				X	X	X	X	X	X	
0.8	X	X	X	X				X	X	X	X	X		
0.9	X	X	X					X	X	X	X	X		
0.9	X	X	X	X				X	X	X	X			
0.9	X	X	X	X	X			X	X	X	X	X	X	
0.9	X	X	X	X				X	X	X	X	X		
1.0	X	X	X	X				X	X	X	X	X		
1.0		X	X	X				X	X	X	X	X		
1.1	X	X	X	X	X			X	X	X	X	X	X	
1.1	X	X	X	X				X	X	X	X			
1.1	X	X	X	X	X			X	X	X	X	X	X	
1.2		X	X	X	X			X	X	X	X	X	X	
1.2	X	X	X	X	X			X	X	X	X	X	X	
1.3	X	X	X	X				X	X	X	X	X	X	X
1.4	X	X	X	X	X	X		X	X	X	X	X	X	X
1.5	X	X	X	X	X			X	X	X	X	X	X	
1.7	X	X	X	X				X	X	X	X			
2.4	X	X	X	X	X			X	X	X	X	X		
2.6	X	X	X	X		X		X	X	X	X			
3.6	X	X	X	X	X	X	ns	X	X	X	X	X	X	ns
4.7	X	X	X	X	X	X		X	X	X	X	X		
Percentage detected	73	87	94	81	39	13	0	100	100	100	100	87	55	17

NS – no sample available.

Table 2. Comparison of detection protocols based on samples collected at weeks 4, 6, 8 and 9

Criterion	Positives detected (%)	Type I errors or false positives (%)	Percentage detected of samples with initial T/E <= 0.2 (%)	Comments
T/E > twice initial value	100	0	100	Only applicable if normal values available
T/E > 4	84	2	13	Current WADA criterion
T/E > 6	72	1	13	Criterion prior to 2005
T/LH > 6 μg/IU	72	4	40	Misses many low T/E samples
LH adjusted for SG < 2 IU/L	77	2	93	Sensitivity less than 50% in week 4
T/E > 4 or LH adj. SG < 2 IU/L	96	6	93	Most sensitive screening procedure
T/E > 6 or LH adj. SG < 2 IU/L	93	4	93	Most cost-effective screening procedure

Adjusted LH results from very dilute urines (SG < 1.005) are to be treated with caution.

administration particularly for those with naturally low T/E values.

A summary of the results obtained from the various detection protocols is presented in Table 2. The samples from the collections made at weeks 4, 6, 8, and 9 were used in the comparison and the Type I error or false presumptive positive rates were calculated from the three samples collected from each subject prior to the testosterone administration. The data show that the incorporation of urinary LH measurement to the steroid profile makes the detection of testosterone doping more effective. Unfortunately the previously proposed use of elevated T/LH values for detecting testosterone doping is the least effective at detecting those with

low natural T/E values. The simplest protocol, which is merely measuring the urinary LH and adjusting for specific gravity, is the most effective overall, selecting 77% of the positive samples. It has an acceptable false presumptive positive rate of 2% using an adjusted cutoff of 2 IU/L. If the two criteria of T/E $>$ 4 and suppressed LH are combined, with either being a trigger for further investigation, the detection rate rises to 96% with a false positive rate of 6%. Raising the T/E cutoff back to the pre-2005 level of six reduces the false positive rate with very little effect on detection sensitivity. We believe that this is the first simple screening protocol that can, without individual based reference ranges, detect those with naturally low T/E values who abuse testosterone. There is also an additional benefit to the measurement of LH in urine relating to the elimination of false presumptive positives. As mentioned previously, approximately 2% of male athletes have natural T/E values of four or above. Adoption of the combination of low LH or T/E $>$ 4 will result in twice the number of false positives, which would be undesirable. However the LH measurements can be used to eliminate many samples that have T/E values above four. It can be seen in the results from the placebo group in Figure 3 that LH values below four are uncommon in normal subjects. Similarly LH values above four are unusual in testosterone-treated subjects, with only 7% of samples collected between weeks 4 and 10 being above four. In the period from six to nine weeks only one sample from the treatment group, other than those from a subject who was also taking finasteride, had an adjusted LH value above four. Thus if a sample received in the laboratory produces a T/E ratio above four but below eight and an adjusted LH value above 4 IU/L (as measured with a DPC Immulite) then this sample should be regarded as normal unless there are other indicators for suspicion. The adoption of this simple measure should markedly reduce the cost of unnecessary additional testing, which is currently being carried out on samples from subjects with naturally elevated T/E ratios. The cost of carrying out a urinary LH measurement is approximately six dollars.

Care should be taken in choosing which assay to use for the measurement of urinary LH as the results that we have found here for normal subjects are very much higher than those previously reported using different assays.[27,28] It has also been reported that urinary LH measurements made using a Delfia immunoassay are consistently lower than those made using the Immulite assay.[29] These differences may reflect epitope variation on the LH molecule or different responses to urinary LH fragments in urine. In either case it will be necessary to compare and harmonize methods for measuring LH in urine if such measurements are to be used successfully for detecting doping with testosterone. Careful consideration should be given to the treatment of the urine prior to LH measurement as it has been reported that LH values are consistently lower in samples from which the urinary sediment has been removed.[29] Other studies have also reported losses of proteins such as hCG and EPO on urine precipitates.[30,31,32]

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