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Reporting and managing elevated testosterone/epitestosterone ratios – Novel aspects after five years' experience

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The testosterone/epitestosterone (T/E) ratio was implemented as an indirect parameter for the detection of testosterone administration with an empirically established threshold value at T/E = 6. In 2005, the T/E reporting threshold was lowered from six to four.

Between 2005 and 2009, 63 510 doping control urine samples were analyzed in the Cologne laboratory. A total of 1442 specimens (2.3%) showed a T/E > 4; 80 (5.5%) of which were tested positive by means of isotope ratio mass spectrometry (IRMS); and most of which (68) originated from strength sport disciplines.

Specimens of high T/E ratio showed a much higher probability for being confirmed to contain exogenous testosterone using IRMS analysis than samples of low T/E values.

Considering the small number of adverse analytical findings triggered by lowering the T/E reporting threshold (978 urine specimens with T/E ratios between 4 and 6 yielded only 4 (0.4%) positive IRMS findings) and the known limitations of the T/E ratio as discriminating parameter (UGT2B17 polymorphism), the currently mandatory approach shows only marginal overall efficiency.

A more effective tool for the detection of the misuse of testosterone would be the implementation of individual reference ranges. Until athlete steroidal passports are available, it is suggested to exceed the threshold level for T/E from 4 to 6 and perform obligatory IRMS analysis for specimens showing T/E > 6. Further conditions triggering IRMS analysis could be suppressed luteinizing hormone (LH) values in males and disproportionate changes of relevant parameters in individual profiles evidently not resulting from ethanol consumption.

Keywords: sport; doping; urine; testosterone; T/E ratio

Introduction

In 1982, the test adopted by the International Olympic Committee (IOC) for the detection of testosterone (T) administration was based on the urinary testosterone/epitestosterone (T/E) ratio.[1] The T/E laboratory reporting threshold was derived empirically from an observed distribution of measurements in specimens collected from a large number of individuals and established at T/E = 6. With an adverse finding, it was mandatory to investigate the T/E results from previous and subsequent tests; i.e. assessing the T/E ratio intra-individually. The reason that elevated T/E ratios need a follow-up before they are declared an adverse finding is the occurrence of naturally elevated T/E ratios.[2]

Since 2005, the World Anti-Doping Agency (WADA) has changed the reporting threshold for elevated T/E values from 6 to 4 in order to improve the sensitivity for the detection of T misuse.[3] T/E values above 4 had to be reported as an adverse analytical finding (AAF) by accredited laboratories to the testing authority, the international federation, and WADA.

In 2008, the term 'atypical finding' was implemented[4] for the reporting of specimen showing a T/E ratio greater than 4, where no determination of the exogenous origin of the target analyte (by means of an additional reliable method, for example, gas chromatography-combustion-isotope ratio mass spectrometry, IRMS) was possible. A time-consuming analytical determination[5] of each single T/E finding greater than 4, enclosing the confirmation of the T/E ratio in triplicate, the verification of the T identity, and the calculation of the relative rate of unconjugated T to T-glucuronide, as well as the retrospective and longitudinal steroid profile evaluation was mandatory for the reporting and management of 'atypical findings'.[6]

In 2010, again changes concerning the management of elevated T/E ratios came into effect,[7,8] leading to a more feasible procedure. No further collection or analysis is now required in cases where the T/E ratio is greater than 4 and an IRMS test or any other reliable analytical method has not revealed evidence of exogenous administration or a prohibited substance. The term 'atypical finding' is no longer relevant for reporting any finding.

Regarding utilization of the T/E ratio as a diagnostic tool for the detection of exogenous T, several items have to be considered. Urinary T/E ratios exceeding 4 may result from an increased excretion of T-glucuronide or a decreased excretion of epitestosterone (E) glucuronide. The application of oral contraceptives in females was observed to lead to an increase of the T/E ratio due to a suppressed E excretion. After cessation

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of the birth control pill, the excretion of E increased, leading to a decreased T/E ratio.[9] On the other hand, genetic aspects of the E formation and influence of polymorphism in CYP17 enzyme were discussed by Schulze *et al*.[10] The presented data indicate 5-androstene-3β, 17α-diol as an important precursor of E, with the cytochrome gene CYP17 involved in its production.

The genotype-dependent variation of the T/E ratio has been shown to result from the UGT2B17 polymorphism.[11] T/E ratios vary among athletes of different ethnic origin. Typically, Asian people have lower urinary T/E values ($<$0.5) than Caucasians (approximately 1.0)[12] and, thus, androgen doping exerts weaker effects on the T excretion in the Asian population, increasing the risk of false-negative results.[13] The lack of the UGT2B17 gene, the active part for the androgen glucuronidation, leads to a low T excretion and consequently to low T/E ratios.[11]

Goebel *et al*. developed a simple screening test to distinguish athletes whose natural T/E values exceed four from those whose T/E values have been elevated by T doping as well as to detect athletes with naturally low T/E values that do not exceed four despite being administered T.[14] The analysis of luteinizing hormone (LH) in all urine samples collected from males and use of the LH concentration together with the T/E ratio as the primary means of screening for doping with T is proposed. Suspicious samples would then be further tested using confirmatory techniques such as IRMS to allow adverse analytical findings to be reported. This methodology is not directly applicable to females because the application of oral contraceptives also suppresses LH secretion.[15]

For the detection of T misuse, the application of individual reference ranges is regarded as a reliable tool. This item was first discussed by Donike *et al*. in 1992.[16–18] It was demonstrated that subject-based reference ranges react sensitively to variations caused by different (pharmacological) interventions and provide a better doping control approach than the ones utilizing population-based reference ranges. Results of longitudinal urinary steroid profile studies[19–23] outlined the low variability within the biosynthesis of endogenous steroids and that the metabolic pathway is in agreement with the stationary, homeostatic model for calculating subject-based reference ranges. In agreement with Donike *et al.'s* publications,[16,17] Sottas *et al*.[24] improved this method by proposing the Bayesian screening test[25] for the detection of abnormal values in longitudinal biomarkers. This test compares sequential measurements of a biomarker against previous readings performed on the same individual. Schulze *et al*.[26] increased the sensitivity and specificity of the test by addition of UGT2B17 genotype information in the Bayesian framework. In addition, Pozo *et al*.[27] suggested the implementation of endogenous T metabolites, analyzed by means of liquid chromatography tandem mass spectrometry (LC-MS/MS) for further improvement of the method.

Recently, Sottas *et al*.[28,29] presented the implementation of the Athlete Biological Passport (ABP) based on the Athlete Steroidal Passport (ASP) for the detection of steroid doping. The ASP, consisting of a longitudinal follow-up (composed of concentration levels of seven endogenous steroids in urine and their respective ratios) together with the ethnicity and/or genotype, would strongly enhance the detection of T abuse.

The oral intake of ethanol can increase the T/E ratio and decrease the androsterone(A)/T ratio by an elevated excretion of T-glucuronide and decreased elimination of A-glucuronide.[30,31] This effect was found more pronounced for females than for males, whereas the changes in the steroid profile ratios were always connected with the presence of ethanol in urine.[32]

Große *et al*. presented ethylglucuronide (EtG) as a suitable marker for alcohol-induced elevation of urinary T/E, which is easily detectable via LC-MS/MS.[33] EtG is slowly excreted into the urine and indicates alcohol intake for a much longer time period than blood or urinary alcohol.

Based on data obtained from the analysis of numerous doping control samples over five years from 2005 to 2009, a revision of the current strategy for the detection of the misuse of T is aimed.

Experimental

Chemicals and reagents

Tert-Butyl methyl ether (TBME) was purchased from KMF Laborchemie (St Augustin, Germany) and distilled before use. ß-Glucuronidase from *Escherichia coli* was supplied by Roche Diganostics GmbH (Mannheim, Germany). N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was obtained from Chem. Fabrik Karl Bucher (Waldstetten, Germany). For gas chromatography/mass spectrometry (GC/MS) analysis testosterone (T) was obtained from National Measurement Institute (via LGC, Wesel, Germany) and epitestosterone (E) was purchased from Sigma (Steinheim, Germany). 17α-Methyltestosterone (Serva, Heidelberg, Germany), [2,2,3,4,4-$^2H_5$]-androsterone glucuronide, [2,2,4,4,-$^2H_4$]-etiocholanolone, [16,16,17-$^2H_3$]-testosterone (d3T) and [16,16,17-$^2H_3$-epitestosterone] (d3-E) (in-house syntheses, Institute of Biochemistry, German Sport University Cologne, Germany) were used as internal standards for the analysis of endogenous anabolic androgenic steroids.[34,35]

For gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS) analysis androsterone (A), etiocholanolone (Etio), pregnanediol (PD) and 5α-androstan-3ß-ol (androstanol, internal standard) were obtained from Sigma (Steinheim, Germany). 5α-Androstan-3α,11ß-diol-17-one (11OHA) was purchased from Steraloids (Rhode Island Newport, USA). Chromabond® C18 cartridges were obtained from Macherey-Nagel (Düren, Germany). All other solvents and reagents were of analytical grade purity.

Sample preparation (anabolic and endogenous steroids)

Common procedures for the detection of anabolic and endogenous steroids in doping controls were applied.[36–38] A volume of 2 mL of urine specimens, independent of gender (3 mL volume is used for urines showing specific gravity less than 1.010) were extracted into 4 mL of TBME at pH 9.6 following enzymatic hydrolysis at pH 7 with β-glucuronidase from *E.coli*. After derivatization with MSTFA/NH_4I/ethanethiol an aliquot of the sample was injected into the GC-MS system.

Gas chromatography/mass spectrometry (GC/MS)

Analyses were performed using a HP 5973 quadrupole mass spectrometer coupled to an HP 6890 gas chromatograph. A J&W Scientific Ultra I (OV-1) column was employed; length 17 m, I.D. 0.2 mm, film thickness 0.11 µm, helium carrier gas at a head pressure of 12 psi.

A 3 µL aliquot of the sample was injected into the GC system which was operated in the split (1 : 10) mode. The GC temperature was ramped as follows: initial temperature 181 °C, program rate 3 °C min^{-1} to 230 °C, program rate 40 °C min^{-1} to 310 °C, keep

constant for 2 min. The injection port and transfer line were heated to 300 °C.

The steroid trimethylsilyl (TMS) derivatives were analyzed using selected ion monitoring (SIM) with electron ionization (EI). Seven groups, containing between 18 and 25 ions, were recorded with scan cycle times between 1.14 and 1.57 s. T and E were registered in groups 3 and 4 (11.4 to 14.6 min) using the quantifier ion at *m/z* 432.2 (20 ms dwell time).

Calculation of the T/E ratio

The estimation of the T/E ratio was performed by means of the internal standards d3T (90 ng/mL) and d3E (15 ng/mL). After adjusting the peak areas for isotopic interference according to Nolteernsting *et al*.,[39] the final T/E ratio was calculated utilizing the d3T/d3E (area) ratio for the correction.

An aliquot of quality control urine (originating from a large volume of urine collected from a healthy male volunteer) is prepared with each batch of doping control urine samples and analyzed at defined frequency within the specimens. The T/E ratios of the quality control urine are collected and monitored.

Performance data for the T/E determination

Performance data for the determination of T/E ratios in screening analysis were prepared following WADA guidelines.[40]

Precision

The assay precision was calculated from the T/E ratio of quality control samples, analyzed in triplicate within one batch of urine specimens in five consecutive months. The precision was determined with 2.1%.

Traceability

The traceability provides an inter-laboratory method performance data approach. The comparison of the consensus T/E value obtained from 32 participating laboratories with the mean value of the T/E determination results in a traceability of 7.2%.

Other sources

The purity of the reference material (as indicated in the standard certificate), as well as estimated errors for standard dilution and sample pipetting is summarized to an uncertainty of 2.29%.

Uncertainty

The combined standard uncertainty (u_c) is calculated from the uncertainty values of precision, traceability and other sources with 7.5%. The extended uncertainty, using a coverage factor $k = 2$, results in 15%.

Sample preparation (GC/C/IRMS)

The extensive sample preparation is based on different purification steps including solid phase extraction (SPE) and separation of the free steroid fraction followed by the hydrolysis of the steroid glucuronides and additional fractionation via high performance liquid chromatography (HPLC), as described in detail elsewhere.[41,42] Depending on the urinary steroid concentration, 2–20 mL of urine are utilized for the sample preparation.

11β-Hydroxyandrosterone (OHA), pregnanediol (PD) and dehydroepiandrosterone (DHEA) are commonly used as endogenous reference compounds (ERC). For screening purposes androsterone (A) and etiocholanolone (Etio) are used as target analytes. In confirmation analysis additionally testosterone (T), 5α-androstane-3α,17β-diol (Adiol) and 5β-androstane-3α,17β-diol (Bdiol) serve as target compounds.

Gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS)

The system used was a HP 5890 gas chromatograph coupled to a Delta C gas isotope ratio mass spectrometer from ThermoElectron (Bremen, Germany) by a combustion interface II (ThermoElectron). The GC was equipped with an OPTIMA-δ 3 analytical column (length 20 m, I.D. 0.25 mm, film thickness 0.25 µm, helium carrier gas at constant flow of 2.2 mL min^{-1}). The injection volume was 2 µL in cool on column mode. The GC temperature was ramped as follows: initial temperature 50 °C, program rate 30 °C min^{-1} to 250 °C, program rate 2 °C min^{-1} to 275 °C, program rate 15 °C min^{-1} to 295 °C, keep constant for 2 min. The GC combustion interface was used with an oxidation reactor temperature of 940 °C.[43]

Urine samples

Between 2005 and 2009, 63 510 doping control urine samples were analyzed in the Cologne doping control laboratory, originating from national and international federations, in-competition (IC) and out of competition (OOC); 16 297 specimens were collected from females and 44 079 from males. For 3134 samples no sex was declared in the covering letter. The specimens were stored at −20 °C for 90 days after reporting and thereafter disposed.

Results and Discussion

One thousand, four hundred and forty-two specimens (2.3%) showed T/E ratios greater than 4, representing 1.4% female and 2.8% male athletes (Figure 1, Table 1), originating from nearly equal parts IC and OOC controls (Table 1). Samples with slightly elevated T/E ratios above 4 are frequently found in endurance and game sport disciplines, whereas strength sport disciplines dominate in urine samples with high T/E ratios (Tables 2 and 3).

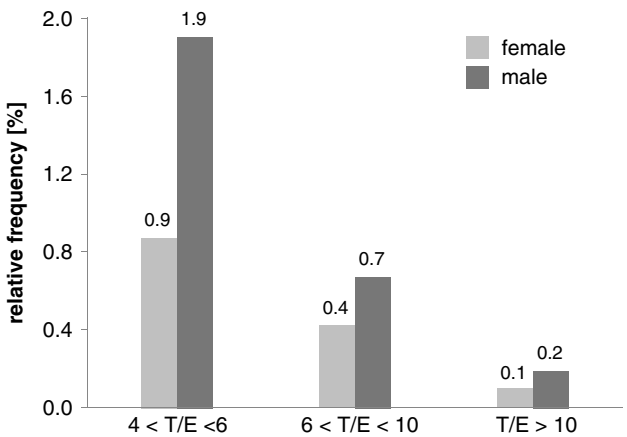

Figure 1. Relative frequency of T/E > 4 (2005–2009).

Table 1. Number of T/E > 4 (2005–2009) in female and male doping control urine samples and resulting adverse analytical findings (AAF), confirmed by means of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Distribution between in-competition (IC) and out-of-competition (OOC) controls

	Female (n = 16,297)				Male (n = 44,079)			
	IC	***AAF (IC)***	OOC	***AAF (OOC)***	IC	***AAF (IC)***	**OOC**	***AAF (OOC)***
n (4 < T/E < 6)	71	***0***	71	***1***	476	***2***	360	***1***
n (6 < T/E < 10)	26	***0***	42	***1***	139	***9***	158	***2***
n (T/E > 10)	9	***5***	8	***4***	43	***30***	39	***25***
∑	106	***5***	121	***6***	658	***41***	557	***28***

Table 2. Number of findings with T/E > 4 in female and male doping control urine samples (2005–2009), confirmed by means of gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS). Distribution between different sport disciplines

	Female								Male							
	Endurance		game		Strength		misc		endurance		game		strength		misc	
	total	***AAF***	total	***AAF***	total	***AAF***	total	***AAF***	total	***AAF***	total	***AAF***	Total	***AAF***	total	***AAF***
n (4 < T/E < 6)	51	***1***	29	***0***	24	***0***	36	***0***	269	***0***	272	***0***	183	***3 (1)***	112	***0***
n (6 < T/E < 10)	23	***1***	15	***0***	11	***0***	19	***0***	91	***0***	86	***0***	91	***9 (2)***	29	***2 (1)***
n (T/E > 10)	5	***1***	0	***0***	10	***8 (4)***	2	***0***	6	***3***	0	***1***	63	***48 (28)***	4	***3 (1)***

(number of multiple positive cases in brackets).

Table 3. Schedule of populations and corresponding sport disciplines

Population	Sport discipline
Endurance	cycling, triathlon, rowing, canoe, speed skating, swimming
Game	soccer, handball, basketball, hockey, icehockey, volleyball, korfball, rugby, tennis, baseball
Strength	weightlifting, powerlifting, wrestling, boxing, bodybuilding, judo, taekwondo, karate
Misc	athletics, shooting, bobsleigh, dance sports, archery, fencing, sailing

From the following presented AAF, the exogenous origin of the T metabolites was proven by means of GC/C/IRMS. Findings of a T/E ratio exceeding the threshold of 4 from the years 2005 to 2007 (which had to be reported as AAF), without evidence of the exogenous origin of the T metabolites, were not enclosed.

Eighty specimens showed positive IRMS results, most of them originating from strength sport disciplines like weightlifting, powerlifting, wrestling, boxing, and bodybuilding (Tables 2 and 3). Specimens of high T/E ratio showed a much higher probability for being confirmed to contain exogenous testosterone using IRMS analysis than samples of low T/E values: 978 urines with T/E between 4 and 6 delivered only 4 (0.4%) positive IRMS findings, 365 specimen yielding T/E between 6 and 10 showed 12 (3.3%) adverse analytical IRMS results and in 64 (65%) of 99 samples having T/E higher than 10, the exogenous origin was proven by means of IRMS (Table 1). Fourteen percent (11 cases) of the adverse analytical findings originated from female athletes: 5 IC and 6 OOC tests (Table 1). The majority of 69 findings came from the male population, representing 41 IC and 28 OOC controls (Table 1). Mainly the adverse analytical findings from strength sport disciplines showed also multiple positive results for different synthetic anabolic steroids (Table 2).

For the detection of T misuse the following important facts have to be considered:

1. Approx. 2% of subjects show T/E ratios above 4 as a usual part of the population,[44–46] leading to unnecessary investigations. Evaluation of steroid profiles based on population reference ranges reveal limitations. The application of individual reference ranges, as discussed by Donike,[16,17] is the basis for a more reliable tool for the detection of T administration. Individual-based evaluations (also known within the anti-doping community as passports) using the Bayesian approach would strongly enhance the detection of T misuse.
2. The genotype-dependent variation of T/E reveals a group of low mode T excretors, which do not produce T/E higher than 4, even after administration of T,[47] resulting in false negative findings. As suggested by Goebel *et al.*,[14] the appropriate measurement of urinary LH, can markedly improve the efficiency of detection of doping with T by male athletes, particularly those who have low natural T/E ratios.
3. The impact of ethanol consumption of the T/E ratio is well known[30–32,48] and should be taken into account when utilizing subject-based reference ranges and longitudinal profiles as tool for the detection of T misuse. Owing to the ease of ethylglucuronide determination procedures, which may potentially be introduced into routine screening, the inclusion of this marker into the final evaluation of suspicious outliers in T/E ratio longitudinal studies seems to be very useful.[33]

Conclusion: Change of Strategy for the Detection of the Misuse of Testosterone

Regarding the small number of adverse analytical findings triggered by lowering of the T/E reporting threshold and the known

limitations of the T/E ratio as discriminating parameter (UGT2B17 polymorphism), the currently mandatory approach shows only marginal overall efficiency.

Based on the evaluated data, an alteration of the strategy for the detection of the misuse of T is proposed. The most effective tool is the implementation of individual reference ranges. Until athlete steroidal passports are available, it is suggested to exceed the threshold level for T/E from 4 to 6 and perform obligatory IRMS analysis for specimens showing T/E $>$ 6. Further conditions triggering IRMS analysis are suppressed LH values in males and disproportionate changes of relevant parameters in individual profiles not due to ethanol consumption.

The modification of the threshold level from 4 to 6 may lead to a slightly increased number of false negative results. However, few false negative results may be acceptable considering the waste of working time and money resources for needlessly carried out confirmatory analysis for a multitude of samples. The investment of these saved resources into LH measurement or randomly performed IRMS analysis (particularly in UGT2B17 polymorphism samples) seems to be a more meaningful tool for the detection of the misuse of testosterone.

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