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Steroid profiles in different ethnic groups of soccer players. A contribution to the determination of testosterone misuse in sports

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Introduction

Testosterone (T) is a steroid hormone naturally produced in the human body commonly used by athletes to enhance sports performance. A T/E ratio exceeding 4.0 is considered as suspicious of T administration, irrespectively of individual heterogeneous factors such as the athlete's ethnicity[1]. The sensitivity of unique, nonspecific thresholds for the detection of T misuse is known to be low.[2,3] Since the T/E ratio has a much higher inter- than intra-individual variability,[4] it is not surprising that subject-based thresholds obtained from a longitudinal follow-up of the steroid profiles lead to a dramatically higher sensitivity.[3] In addition, studies with Caucasian and Asian populations pointed out significant differences in the excretion of T glucuronides.[5,6,7] It was demonstrated that the bimodal distribution of urinary T concentrations is associated to a deletion mutation in the UGT2B17 gene.[8] Noteworthy, subjects deficient in this gene never presented a T/E value higher than 4.0 following administration of testosterone.[9,10]

In this study, the magnitude of urinary steroid levels and their respective ratios is examined in a cohort of 171 top-level soccer players. The range of the carbon isotope ratio of the steroids relevant to anti-doping analysis will be investigated as well. The determination of threshold values specific to steroid profiling and to isotope analysis is expected to significantly improve the detection test of T misuse.

Experimental

Studied population and samples collection

Urine samples were collected from 171 male soccer players aged from 18 to 36 (mean age: 24.5 ± 3.8 yr). These athletes were residing in Argentina (ARG, n = 31), Italy (ITA, n = 19),

Japan (JAP, n = 32), Republic of South Africa (SAF, n = 30), Switzerland (SWI, n = 31) and Uganda (UGA, n = 28). They were divided into the following groups: African (AF, n = 57), Asian (AS, n = 32), Caucasian (CA, n = 50) and Hispanic (HI, n = 32). The study design has been evaluated and accepted by the ethical committee of the University of Lausanne (Switzerland). The specimens were not collected during regular anti-doping tests.

Sample preparation

Urinary steroid profiles were determined by GC-MS according to a routine screening procedure developed for anti-doping testing.[11]

For the GC-C-IRMS analysis, a validated method was used to determine the carbon isotope ratio of androsterone (A), etiocholanolone (Etio), 16(5α)-androstenol (16EN) and 5β-pregnanediol (PD).[12] It is noteworthy that no drift was observed in the carbon isotope ratio of the analytes in the QC during the period of analysis (5 months) and the standard deviations reached 0.39, 0.34, 0.34 and 0.29 ‰ for Etio, A, 16EN and PD, respectively.

Results and discussion

The analyses of all samples revealed that no steroid profile presented an absolute steroid concentration higher than its respective threshold defined by WADA, while 3 samples with a T/E > 4 (1.7%) were measured. Nevertheless, systematic GC-C-IRMS investigations on all 171 specimens did not return any result consistent with the administration of an exogenous steroid (data not shown).

The urinary steroid concentrations and ratios levels classified following the ethnic group of the soccer players are illustrated in Figure 1. The median values of T/E ratio (with 25th and 75th percentile) were of 0.8 (0.4-1.4), 0.2 (0.1-0.3), 1.1 (0.6-2.0) and 1.2 (0.8-2.1) for AF, AS, CA and HI, respectively. It was significantly lower for AS than in the other ethnic groups ($p = 0$) and higher in HI compared to AF ($p = 0.04$). Among all ethnic groups, the major influence on T/E ratio originated from the variations in T concentrations for which the median was of 40.5 (9.5-72.2) ng/mL, while that of E was of 42.9 (26.9-68.8) ng/mL. Several comparative studies of urinary T levels among ethnic groups have been reported in the litterature.[6,8] In agreement with our investigation, a larger number of CA athletes excrete more T in urine than AS athletes. In contrast, CA populations display a significantly lower E level than AF ($p = 0$) while AS release lower amounts of E in urine than AF and HI ($p < 0.02$). It is also important to note that, AF and HI groups show significant higher DHEA glucuronide concentrations than AS and CA populations ($p = 0$).

To rationalize these findings, an additional polymorphism may be hypothesized. For instance, it is already known that a polymorphism on the cytochrome P-450c17α (CYP17) partially contributes to the variations observed in the phase I metabolism.[13] Actually, it may not be derived from our experimental design whether the differences in the production of androgen glucuronides might be due to environmental/dietary or genetic factors.

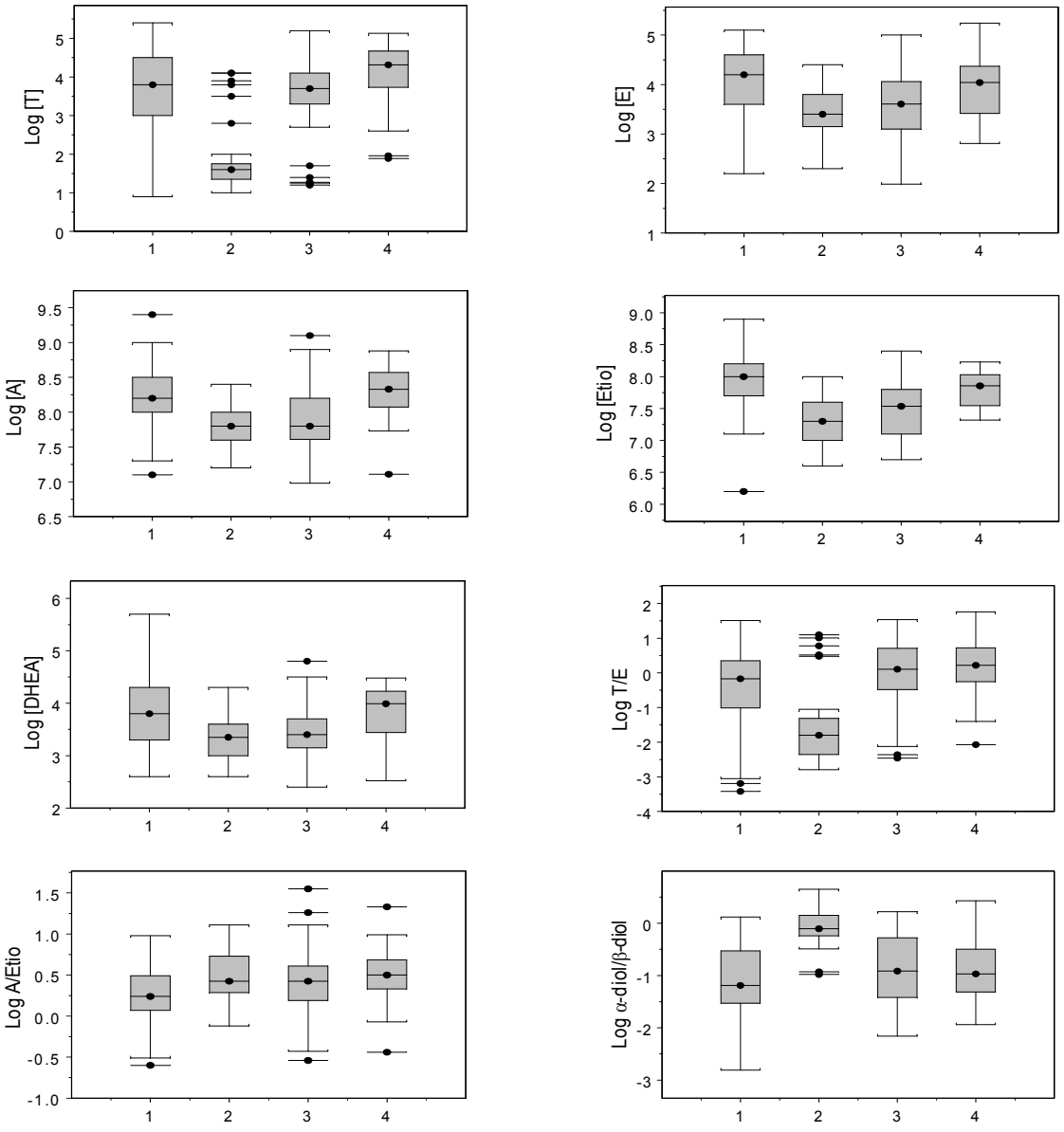

Figure 1: Urinary levels of glucuroconjugated steroids (log ng/mL) for testosterone, epitestosterone, androsterone, etiocholanolone and DHEA and ratios (expressed as log) of T/E, A/Etio and α-diol/β-diol in African (1), Asian (2), Caucasian (3) and Hispanic (4) populations.

To evaluate the possibility that environmental/dietary factors would contribute significantly, different ethnic groups composed of a homogenous number of subjects among any of the selected countries should be tested.

The prevalences of the UGT2B17 deletion polymorphism for each ethnic group, estimated from the phenotypic results, were of 22%, 81%, 10% and 7% for AF, AS, CA and HI, respectively. It could be related to the prevalences estimation found in the literature.[8,14,15] The significant variations for the same ethnic group depends certainly on the ethnic group definition. Our subjective ethnic classification seems too rudimentary owing to a significant difference in the level of T between athletes from Uganda and South Africa ($p = 0$) (data not shown). A study including more subjects should be performed to strictly set apart close ethnic groups. Xu et al. have described a more detailed distribution of the UGT2B17 deletion polymorphism in the world population.[16] Interestingly, in our study, the UGT2B17 deletion prevalence in HI from Argentina (7%) is in full agreement to that found for CA (10%). This observation contrasts with prevalence values of 50% and 73% reported for HI subjects from Brazil and Colombia, respectively.[17]

We added the prevalence estimates of the UGT2B17 deletion as prior information in a Bayesian model to give an order of magnitude of the part of the variance in the T/E ratio explained by the differences in ethnicity.[3] We found ethnic-specific cutoffs of 5.6 for AF, 3.8 for AS, 5.7 for CA and 5.8 for HI, for a specificity of 99%. In comparison, *genotype-specific cutoffs are equal to 0.5 for del/del subjects and 5.9 for ins/del and ins/ins subjects,* the latter initial value of 5.9 decreasing to a value as low as 2.2 with an initial, individual baseline T/E ratio of 1.0. These results suggest that the knowledge of a unique baseline T/E ratio allows a significantly greater individualization of the T/E reference range than the knowledge of the ethnicity of the athlete.

Glucuronide derivatives of A and α-diol are frequently used as indirect markers of 5α-reductase activity,[17] whereas Etio and β-diol glucuronides are indirectly related to 5β-reductase activity. In this study, urinary concentrations of A and Etio were significantly lower in AS and CA groups than in subjects from AF and HI groups ($p < 0.02$). These results tend to confirm the lower excretion of 5α-reduced steroids in Asians.[18,19] To rationalize the data, a gene polymorphism responsible of the 5α-reductase activity was described.[20] Noteworthy, 5α-reductase activity is compared to that of 5β-reductase through the concentration ratios of 5α-/5β- reduced steroids.[7] In our study, the A/Etio ratio values were of 1.3 (1.1-1.6), 1.5 (1.3-2.1), 1.5 (1.2-1.8) and 1.7 (1.4-2.0) for AF, AS, CA and HI,

respectively. It was significantly lower for AF than for AS and HI men ($p < 0.02$). Interestingly, HI A/Etio ratio was not significantly different from that of AS ($p = 0.97$) and CA ($p = 0.52$). The levels determined in urine for α- and β-diol evidence the same differences between ethnic groups than for 17-keto metabolites (data not shown). Low α-diol levels might arise in men who have a lower conversion of dihydrotestosterone to α-diol or, conversely, a higher reconversion of α-diol to dihydrotestosterone.[21] On the other hand, it may be due to a greater efficiency of T conversion to estradiol due to higher aromatase activity (CYP19 polymorphism).17 Median values of the α-diol/β-diol ratio were of 0.3 (0.2-0.6), 0.9 (0.8-1.1), 0.4 (0.2-0.7) and 0.4 (0.3-0.6) for AF, AS, CA and HI, respectively. This ratio was higher for AS than for AF, CA and HI ($p = 0$), thereby showing that 5α-reductase may be more active in AS. Although a relation with the UGT2B17 deletion polymorphism was expected to explain this difference, no correlation could be found between T and the other steroids of interest. Additional studies are required to give prominence to a new polymorphism.

For stable isotope determination, the analysis was performed according to the residence country of each athlete. The $^{13}C/^{12}C$ ratio expressed in $\delta^{13}C$ values (‰) of A and Etio together with 16(5α)-androstenol (16EN) and 5β-pregnanediol (PD) are displayed in Figure 2. Comparisons between the different groups show that Italian and Swiss populations have more negative $\delta^{13}C$ values for all the tested steroids. At the same time, the Japanese athletes exhibited intermediate $\delta^{13}C$ values compared to those of the European countries and the group comprising ARG, SAF and UGA. For all the steroids investigated herein, Argentinean players and players of African countries displayed a similar enrichment in ^{13}C ($p > 0.2$). The $\delta^{13}C$ values measured for the target compounds in each of the 171 urine specimens ranged from -17.2 to -25.2 ‰, thus reflecting the extent of isotopic fractionation as a function of the athletes' diets and metabolisms. In our study, we can assume that no significant shifts in the eating habits of the athletes occurred in the month prior to urine collection. Thus, it may be hypothesized that the excreted steroids were isotopically equilibrated with respect to the athlete's diet. In agreement with the data published recently, the mean $\delta^{13}C$-values measured for A were -19.0, -20.5 and -22.0 ‰ for SAF, JAP and ITA, respectively. This trend which is also confirmed for Etio, 16EN and PD corresponds to an apparent higher consumption of C3-foodstuffs by European populations compared to other countries.

The natural variation in $^{13}C/^{12}C$ composition of steroids excreted in urine was recently described for male and female subjects residing in Germany.[22] For this European population,

a metabolic ^{13}C fractionation was observed between A and Etio. It is believed that the isotopic fractionation between both 5α- and 5β-metabolites originates from a kinetic isotope effect during the reduction step of T.[23] Our data show significant ^{13}C depletion in Etio compared to A for ARG, JAP, ITA and SWI populations ($p < 0.02$). In contrast, both T metabolites display comparable ^{13}C enrichment for the athletes of SAF and UGA.

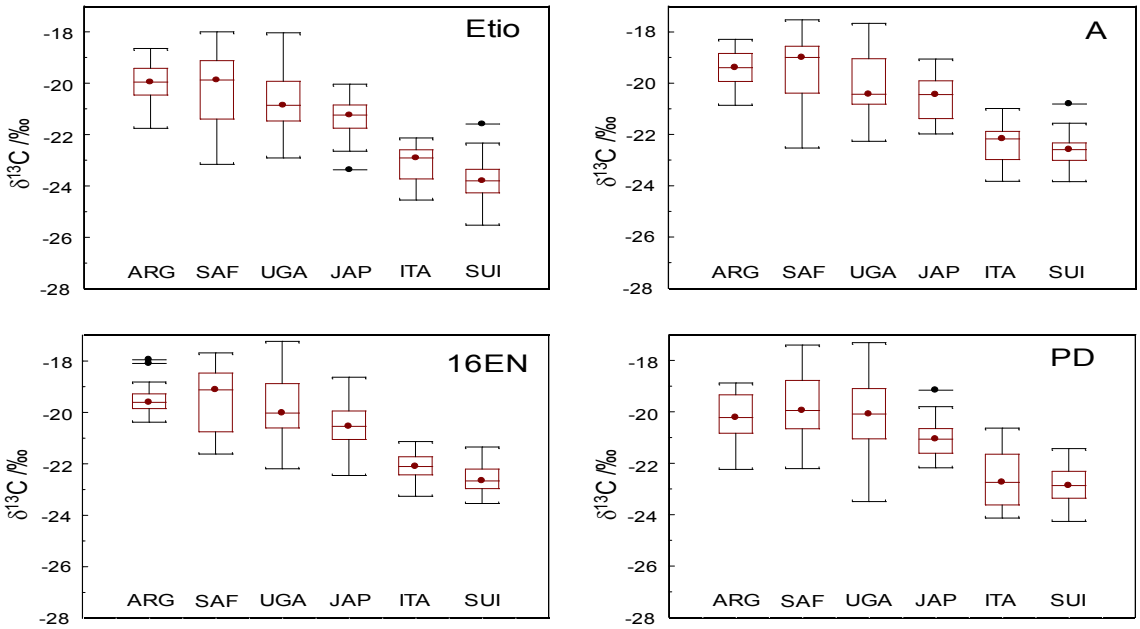

Figure 2: Distribution of $\delta^{13}C$-values obtained for etiocholanolone (Etio), androsterone (A), 16(5α)-androstenol (16EN), and 5β-pregnanediol (PD) in the urine specimens of the top-level soccer players residing in the 6 surveyed countries.

According to WADA guidelines, a $\Delta\delta^{13}C$ difference of 3.0 ‰ or more between the $\delta^{13}C$ values of T metabolites and endogenous reference compounds (ERC) is consistent with the administration of exogenous steroids.[1] This biomarker derived from the difference between isotope ratios constitutes a corrective factor for one's individual diet. Importantly, the technical document is applicable irrespective of the choice of the T metabolite and the ERC chosen to calculate the $\Delta\delta^{13}C$ value. In our study, it appears that the isotopic fractionation between androsterone and the ERCs is comparable for all individuals, regardless of their diet habit. Indeed, in the different groups of soccer players, no statistical difference was found between the $\Delta\delta^{13}C$ values calculated from the difference between the $\delta^{13}C$ values of androsterone and 16(5α)-androstenol or 5β-pregnanediol. In agreement with these findings, Piper et al. observed no significant change in the difference between the $^{13}C/^{12}C$ ratios of

11β-hydroxyandrosterone, a product of the cortisol-cortisone metabolic pathway, and androsterone in urine samples of athletes residing in various geographical regions of the world.[24]

Table 1 lists the 99% confidence intervals of the differences in $^{13}C/^{12}C$ composition between testosterone metabolites and the ERCs for each athletes group.

Table 1. Reference limits of the $\Delta\delta^{13}C$ values for each surveyed country calculated by the addition of the mean value and 3-fold standard deviation. All values are in $\delta^{13}C_{VPDB}$ (‰).

	Reference limit in ‰			
	Δ(16EN-Etio)	Δ(16EN-A)	Δ(PD-Etio)	Δ(PD-A)
Uganda (n = 28)	2.8	2.3	3.0	2.3
South Africa (n = 30)	2.5	2.3	3.2	2.4
Switzerland (n = 31)	3.4	1.2	3.2	1.4
Italy (n = 19)	2.9	2.6	3.2	1.8
Argentina (n = 31)	2.6	1.9	2.7	1.7
Japan (n = 32)	3.2	2.9	2.5	2.3

16EN, 16(5α)-androstenol; Etio, etiocholanolone; A, androsterone; PD, 5β-pregnanediol

These intervals are determined by the sum of the mean value and threefold the standard deviation. Our data reveal discrepancies depending on the pair of steroids that is considered, thus illustrating the occurrence of isotope fractionation in steroid metabolism. Specifically, 99% confidence intervals for the differences in $^{13}C/^{12}C$ content between PD and A are systematically lower than 2.5‰ whereas the 3‰ threshold value may not be suitable for steroid pairs based on the $\delta^{13}C$ value of Etio and the ERCs.

Conclusion

The mode of T excretion has important implications in the interpretation of a steroid profile. This study demonstrated that there are significant differences in the steroid profile in a heterogeneous cohort of professional soccer players. Therefore, unique and nonspecific thresholds on absolute concentrations of steroids and their respective ratios are not fit for purpose to evidence T misuse in international sports. An individualization of the reference ranges based on a longitudinal analysis of individual steroid profiles in an athlete's endocrinological passport should be implemented. Since isotopic analysis using GC/C/IRMS demonstrated to be a useful tool to increase the detection efficiency of a T misuse by a low excretor, this technology has to be applied on specimens displaying significant variations of the steroid pattern with respect to individual norms. This method is expected to become a universal analytical test in the forthcoming years. This study assessed the sensitivity of the assay which is limited by the imprecision in the measurements of the carbon isotope ratio and the natural isotope fractionation occurring during the metabolism of androgens.

In agreement with previous studies,[22] our investigation suggests that different thresholds specific for each pair of steroids should be established. Of course, the evaluation of appropriate cut-offs should be assessed though inter-laboratory collaborative trials. Such an approach would significantly increase the power of the test to evidence T misuse.

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