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Case report

Elevated and similar urinary testosterone/epitestosterone ratio in all samples of a competition testing: Suspicion of a manipulation

Neil Robinson [a,*], Vincent Castella [b], Christophe Saudan [a], Pierre-Edouard Sottas [a], Carine Schweizer [a], Noelle Dimo-Simonin [b], Patrice Mangin [a], Martial Saugy [a]

[a] *Laboratoire Suisse d'Analyse du Dopage, Institut Universitaire de Médecine Légale, rue du Bugnon 21, 1005 Lausanne, Switzerland*
[b] *Laboratoire de Génétique Forensique, Institut Universitaire de Médecine Légale, rue du Bugnon 21, 1005 Lausanne, Switzerland*

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Abstract

The case of seven urine samples collected for anti-doping purposes during a cycling stage race with moderately elevated testosterone and epitestosterone ratio (T/E) is reported. The very low probability of having all seven urine samples with such similar elevated T/E ratio (from 3.2 to 4.7) was very suspicious. Different pattern classification tools were tested to categorize the most similar steroid profiles, but none of the models enabled a clear classification of the different urine samples. Subsequently, genetic profiling of all urine samples was performed and demonstrated that three of the seven samples were collected from the same cyclist. Finally, the International Federation confirmed DNA profiling results. This suggests that urinary steroid data using several methodologies are not appropriate for identification purposes and to an extent not unique to individuals.

Keywords: DNA polymorphism; Testosterone; Epitestosterone; Isotope ratio mass spectrometry (IRMS); Doping control; Steroid profile

1. Introduction

Testosterone (T) abuse is widespread among sportsmen willing to increase strength, aggressiveness and recovery [1]. The effectiveness of this anabolic steroid and in particular the difficulty of differentiating both endogenous and exogenous species in urine makes it interesting for abusers. Underground magazines and books promote the intake of this specific steroid, because it can be taken orally, intravenously or subcutaneously according to the detection window and the effectiveness of the drug. Within this context, Donike et al. defined that a urinary testosterone/epitestosterone (T/E) ratio > 6 is an indication of exogenous use of testosterone enhancing compounds, unless there is evidence that this ratio is due to a physiological or pathological condition (e.g. low epitestosterone (E) excretion, androgen producing tumour and enzyme deficiencies) [2]. For healthy men, it has been found that this parameter is fairly steady over months (CV $< 30\%$) and quite typical for each individual [3,4].

In a technical document dated August 2004, the World Anti-Doping Agency (WADA) has reduced the T/E ratio

* Corresponding author. Tel.: +41 21 314 73 30; fax: +41 21 314 70 95.
E-mail address: Neil.Robinson@chuv.ch (N. Robinson).

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down to 4 [5]. Before declaring an athlete with a T/E > 4 positive to exogenous testosterone, longitudinal follow-up and determination of $^{13}C/^{12}C$ ratio of testosterone metabolites have to be conducted.

2. Case history

During a cycling event in 2004, seven urine samples collected consecutively over 3 days were sent to a WADA accredited anti-doping laboratory for routine analyses. All samples were collected in competition and screening analyses were conducted [6]. Unusual and similar T/E ratios were observed for all samples. Due to the very low probability of having that many urine samples with such T/E ratio values during the same competition, the anti-doping laboratory suspected the athletes and/or the person in charge of the urine collection of having manipulated the samples. To discard any doubts on anti-doping controls, the International Cycling Federation (UCI) decided to have all urine samples sent to our laboratory for further investigations.

3. Methodology

Urinary concentrations of testosterone, epitestosterone, androsterone, etiocholanolone, dihydrotestosterone (DHT), 5α-androstan-3α-17β-diol and 5β-androstan-3α-17β-diol concentrations were determined by means of gas chromatography/mass spectrometry (GC/MS). The extraction procedure and chromatographic conditions were published elsewhere [7]. The specific gravity was also measured to adjust for the specific gravity of the urine sample [8].

Carbon isotopic ratio $^{13}C/^{12}C$ of urinary androsterone and etiocholanolone (two metabolites of testosterone) were determined according to the method of Aguilera et al. [9]. We integrated together the signals corresponding to androsterone and etiocholanolone for a better precision on the isotopic measurements [10]. To allow this, it was checked by GC/MS that the peaks of interest were not contaminated with other substances.

Genetic profiling was used to determine whether several urines could come from the same athlete [11,12]. For this purpose, total DNA was extracted from urine samples using a phenol-chloroform protocol [13]. Recovered DNA was then analyzed using either nuclear (SGM Plus kit from Applied Biosystems) or mitochondrial DNA markers (sequencing of the hypervariable regions I and II from the D-loop). Genetic profiles were visualized on an ABI 310 Genetic Analyzer using standard protocols.

4. Results and discussion

All samples were extracted for determination of steroid profile and carbon isotopic ratio (expressed as delta ^{13}C-values) of urinary steroids. Both procedures are performed on a regular basis in most anti-doping laboratories. As shown in Fig. 1, T/E ratios of the seven urine samples are ranging from 3.2 to 4.7 (mean = 3.7, S.D. = 0.6). On the basis of our internal T/E ratio database (male athletes, n = 4885, mean T/E = 1.54, S.D. = 1.32) depicted in Fig. 2, the expected probability of having a T/E ratio between 2.5 and 4.8 (mean ± 2S.D. = 3.7 ± 2 × 0.6) is equal to 0.114 (95% CI = 0.06–0.18, 1000 bootstrap samples [14]). With a collection of seven independent samples, one can expect only one value falling in this range. The probability that all seven

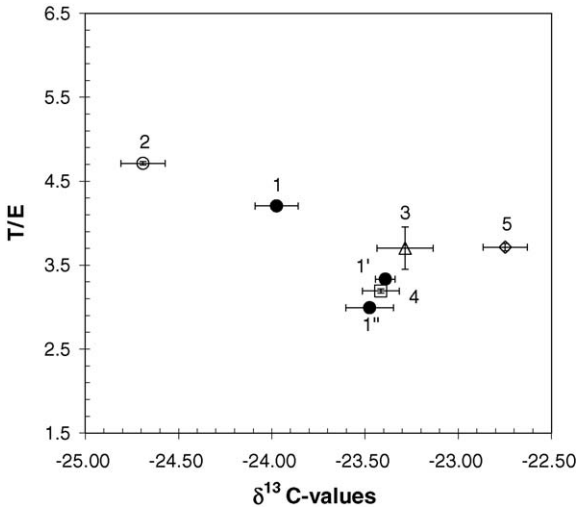

Fig. 1. Two by two representation of T/E ratio together with $\delta^{13}C$-values of all seven urine samples. Identification of the samples based on DNA analyses were performed subsequently. Black dots correspond to data obtained from the same athlete (3 consecutive days), whereas white dot, square, triangle and diamond come from four different persons.

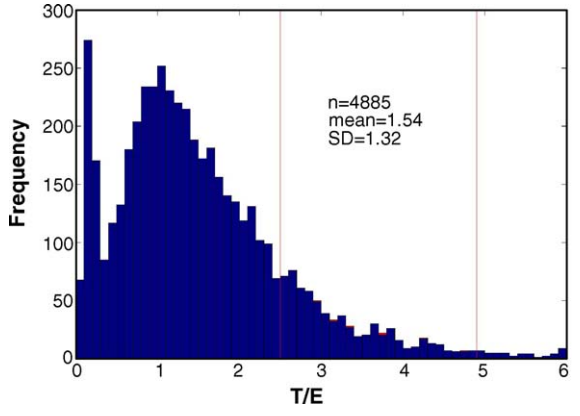

Fig. 2. Internal database showing T/E ratio of professional sportsmen (n = 4885). Vertical lines represent the limits defined by the mean T/E ratio ± 2S.D. observed for all seven urine samples collected during the cycling race.