

Case report

Short term impact of *Tribulus terrestris* intake on doping control analysis of endogenous steroids

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Abstract

Tribulus terrestris is a nutritional supplement highly debated regarding its physiological and actual effects on the organism. The main claimed effect is an increase of testosterone anabolic and androgenic action through the activation of endogenous testosterone production. Even if this biological pathway is not entirely proven, *T. terrestris* is regularly used by athletes. Recently, the analysis of two female urine samples by GC/C/IRMS (gas chromatography/combustion/isotope-ratio-mass-spectrometry) conclusively revealed the administration of exogenous testosterone or its precursors, even if the testosterone glucuronide/epitestosterone glucuronide (T/E) ratio and steroid marker concentrations were below the cut-off values defined by World Anti-Doping Agency (WADA). To argue against this adverse analytical finding, the athletes recognized having used *T. terrestris* in their diet. In order to test this hypothesis, two female volunteers ingested 500 mg of *T. terrestris*, three times a day and for two consecutive days. All spot urines were collected during 48 h after the first intake. The $^{13}\text{C}/^{12}\text{C}$ ratio of ketosteroids was determined by GC/C/IRMS, the T/E ratio and DHEA concentrations were measured by GC/MS and LH concentrations by radioimmunoassay. None of these parameters revealed a significant variation or increased above the WADA cut-off limits. Hence, the short-term treatment with *T. terrestris* showed no impact on the endogenous testosterone metabolism of the two subjects.

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1. Introduction

According to guidance given by the World Anti-Doping Agency (WADA) in 2004, urine samples should be now submitted to isotopic ratio mass spectrometry (IRMS) if the testosterone glucuronide/epitestosterone glucuronide (T/E) ratio is greater or equal to 4.0 and if steroid concentrations corrected with specific gravity are greater than fixed cut-off values: testosterone (T) > 200 ng/mL, epitestosterone (E) > 200 ng/mL, androsterone (A) > 10,000 ng/mL, etiocholanolone (Et) > 10,000 ng/mL and dehydroepiandrosterone (DHEA) > 100 ng/mL [1]. The ratio of the two stable carbon isotopes ($^{13}\text{C}/^{12}\text{C}$) allows the differentiation between natural and synthetic steroids. As exogenous testosterone or precursors contain less ^{13}C than their endogenous homologs, it is expected that urinary steroids with a low $^{13}\text{C}/^{12}\text{C}$ ratio originate from

pharmaceutical source [2]. Endogenous steroids are produced in the body from cholesterol which is derived from an average of a wide variety of vegetal and animal precursors or synthesized from precursors of feed origin.

The method for determining the isotopic composition of the relevant analytes includes gas chromatography, a subsequent combustion to CO_2 and finally, mass spectrometric analysis of this gas in a specific multi-collector mass spectrometer (gas chromatography/combustion/isotope-ratio-mass-spectrometry, GC/C/IRMS) [3]. The $^{13}\text{C}/^{12}\text{C}$ ratio, expressed in $\delta^{13}\text{C}$ values (‰) versus VPDB (Vienna Pee Dee Belemnite), will be determined for testosterone or its metabolites and compared to that of urinary reference steroids within the sample in order to consider variation in athlete's diet and metabolism [4,5]. In addition, it should be emphasized that the $^{13}\text{C}/^{12}\text{C}$ ratio of these endogenous reference compounds should not be affected by steroid administration [6,7]. The result will be reported as consistent with the administration of a steroid if a difference of 3.0‰ or more is determined between the $\delta^{13}\text{C}$ values of testosterone metabolites and endogenous reference compounds

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or if the $\delta^{13}\text{C}$ value of underivatized testosterone metabolite(s) is below -28‰ [1].

2. Case history

With the aim of improving the efficiency of anti-doping controls, targeted testing are conducted regularly by the international federations. In this context, two female athletes were tested positive by a WADA accredited laboratory following the federation no-advance notice and out of competition doping control. The analysis of the urine samples by GC/C/IRMS conclusively established the administration of exogenous testosterone or its precursors, though the T/E ratio and steroid marker concentrations were below the cut-off values defined by the WADA. Similarly to A-samples, all B-samples confirmed that the carbon isotope ratio of androsterone and etiocholanolone were significantly different from the endogenous references chosen. In addition, the ratio measured for etiocholanolone were significantly below -28‰ based on non-derivatized steroid. To vindicate these results, it was argued that the athletes do frequently ingest *Tribulus terrestris*, a plant extract that may contain DHEA precursors.

3. Methods

Two healthy and Caucasian female volunteers (26 and 40 years old) living in Switzerland ingested two 250 mg capsules of *T. terrestris* supplement (Tribestan[®], Sopharma, Bulgaria) three times a day (in the morning, at noon and in the evening) during two consecutive days. The study was in accordance with the Helsinki Declaration of 1975 and all of the subjects gave their written informed consent. Baseline urine samples were obtained before initial administration, and subsequent spot urine samples were collected over a period of 50 h after the first administration. Each urine sample was divided into 20-mL flasks and stored without additives at -20 °C until analysis.

Urinary concentrations of testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Et), 5α -androstane- 3α - 17β -diol, 5β -androstane- 3α - 17β -diol and dehydroepiandrosterone (DHEA) were determined by gas chromatography/mass spectrometry (GC/MS). The extraction procedure and chromatographic conditions were published elsewhere [8]. The analyses were performed in single ion monitoring mode (SIM) with $m/z = 432, 432, 434, 434, 256, 256$ and 432 for T, E, A, Et, 5β -androstane- 3α - 17β -diol and DHEA, respectively. For each substance, a six-point calibration curve was established using available reference material with the following urinary

concentration range: testosterone and epitestosterone ($5\text{--}250\text{ ng/mL}$, $R^2 > 0.996$), androsterone and etiocholanolone ($200\text{--}6000\text{ ng/mL}$, $R^2 > 0.998$), 5β -androstane- 3α - 17β -diol and 5α -androstane- 3α - 17β -diol ($5\text{--}500\text{ ng/mL}$, $R^2 > 0.998$), DHEA ($5\text{--}500\text{ ng/mL}$, $R^2 > 0.997$).

The GC/C/IRMS method and experimental conditions for the determination of urinary $\delta^{13}\text{C}$ -values of $16(5\alpha)$ -androstene- 3α -ol (androstenol), androsterone and etiocholanolone were used with subsequent modifications of the chromatographic conditions [6]. Briefly, chromatographic separations were achieved on a DB-17MS capillary column ($30\text{ m} \times 0.25\text{ mm i.d.}$, $0.25\text{ }\mu\text{m}$ film thickness) from J&W Scientific (Folsom, CA, USA). The injector temperature was set to 280 °C . The combustion and reduction oven temperatures were set to 940 °C and 600 °C , respectively. For the analysis of fraction 1 containing androsterone and etiocholanolone acetates and fraction 2 with androstene- 3α -ol acetate (reference compound), the oven temperature was increased from 70 °C (1 min) to 271 °C at 30 °C/min , then to 281 °C (3.0 min) at 1 °C/min , and finally to 300 °C (5.0 min) at 10 °C/min . The injection volume was $1\text{ }\mu\text{L}$ and the extracts were injected in the splitless mode (1:30 min).

Luteinizing hormone (LH) was measured in every sport urine using the Elecsys[®] 1010 LH immunoassay from Roche (Roche Diagnostics, Rotkreuz, Switzerland) [9].

4. Results and discussion

T. terrestris is an herbal plant that has been extensively used in Chinese and Indian traditional medicine for the treatment of various disorders [10]. It contains protodioscin, a steroidal saponin, that was found to increase the level of testosterone, dehydroepiandrosterone (DHEA) and luteinizing hormone. Based on significant increase of DHEA sulphate levels in the serum of a patient suffering from erectile dysfunction and treated with *T. terrestris*, it was hypothesized that protodioscin was converted into DHEA sulphate into the body [11]. However, well-controlled clinical trial regarding the effect of DHEA or potential precursor of DHEA are actually missing [12].

DHEA is a steroid precursor of testosterone, produced both in the free (DHEA) and sulphated form (DHEA-S), with an interconversion equilibrium between both forms. In a study, it has been shown that about 1.5% DHEA is metabolized into testosterone and that the intake of a single oral dose of DHEA does not increase the T/E ratio in a group of nine healthy subjects [13]. Following guidance given by the WADA, a threshold for corrected concentrations of DHEA glucuronide at 100 ng/mL is used for identification of samples containing

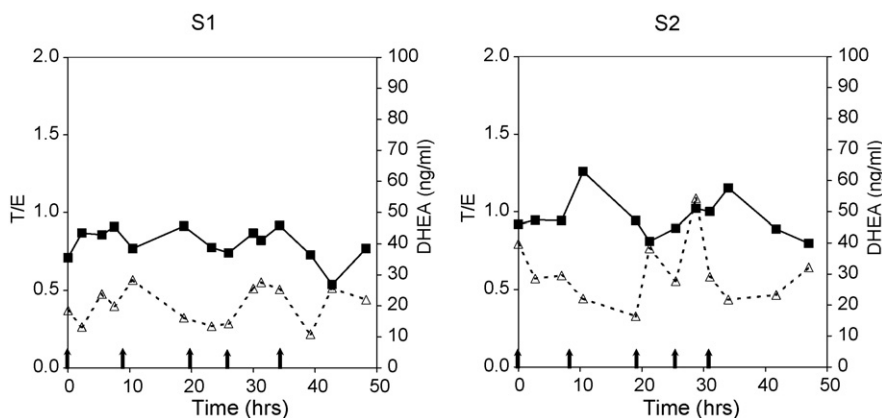


Fig. 1. Urinary T/E ratio (■) and DHEA concentration (△) for subjects S1 (left) and S2 (right). The oral doses of $2 \times 250\text{ mg}$ of Tribestan[®] are indicated by the bold arrows.

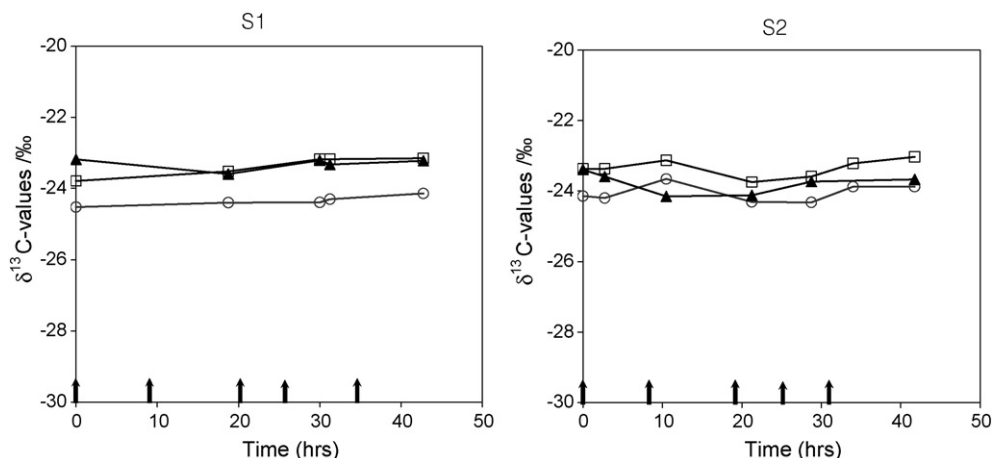


Fig. 2. Urinary $\delta^{13}\text{C}$ -values of androsterone (\square), etiocholanolone (\circ) and androstenol (\blacktriangle) for selected spot urines of subjects S1 (left) and S2 (right). The oral doses of 2×250 mg of Tribestan[®] are indicated by the bold arrows.

exogenous DHEA [1]. Beyond GC/C/IRMS analysis for direct determination of exogenous application of DHEA [6,14], the concentration level of DHEA glucuronide appears to be the most sensitive indirect parameter in the urinary steroid profile to ascertain intake of the substance [13].

Following the hypothesis that protodioscin is a precursor of DHEA, it may be expected that the urinary concentration of DHEA glucuronide would increase and the carbon isotope ratio of urinary androsterone and etiocholanolone (urinary metabolites) would be affected relatively to the isotopic composition and metabolism of protodioscin in the body. To confirm this hypothesis, two excretion studies with an extract of *T. terrestris* (Tribestan[®]) were carried out with multiple doses of the extract during urine samples collection. Fig. 1 shows the DHEA concentration throughout the excretion study together with the T/E ratio. Similarly to previous studies regarding *T. terrestris* supplementation [15,16], these results show no significant longitudinal variation of the urinary steroid profile upon intake of 500 mg of Tribestan[®] three times a day for two consecutive days. More precisely, the concentrations of DHEA in all collected urine samples remained well below the threshold value of 100 ng/mL and the T/E ratio did not vary significantly from the basal values [17] (Fig. 1). In addition, the measured urinary LH concentrations remained below 5 mIU/ml and did not show any significant variation (data not shown).

The carbon isotope ratio of testosterone metabolites (androsterone and etiocholanolone) and the endogenous reference (androstenol) compounds were not affected by intake of the plant extract (Fig. 2). From these results, it is likely that the extract contains no precursor of testosterone or a testosterone prohormone which would result in a variation of $^{13}\text{C}/^{12}\text{C}$ ratio of androsterone and etiocholanolone. This finding complements the results obtained for the T/E and DHEA profiles depicted in Fig. 1. Thus, *T. terrestris* may not be considered either as a direct precursor of testosterone or as a stimulating agent of endogenous testosterone production. However, it may be possible that daily intake of the plant extract over a longer period of time could lead to new equilibrations of the $^{13}\text{C}/^{12}\text{C}$ ratio for endogenous steroids.

Depending on the diet consumption of C_3 - and C_4 - plants, metabolism of nutrients and subsequent *de novo* cholesterol biosynthesis could lead to slow changes of steroid $^{13}\text{C}/^{12}\text{C}$ ratio [18]. As *T. terrestris* is a C_4 -plant species [19], it may be hypothesized that after ingestion, consequent metabolism of this plant would result in a new equilibration of the $^{13}\text{C}/^{12}\text{C}$ ratio of endogenous steroids with ^{13}C enrichment. Again, this would not explain the positive IRMS results found for both top-level female athletes.

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