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INGESTION OF DESIGNER SUPPLEMENTS PRODUCED POSITIVE DOPING CASES UNEXPECTED BY THE ATHLETES

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ABSTRACT: Since the end of the last century, the sport community has been grappling with contamination and faking of dietary or nutritional supplements. Publication of the results of many studies on this problem has resulted in educational and informative actions on risk connected with consumption of unchecked products. As compared to the past, nowadays athletes have become incomparably more aware of the potential risk of a positive result in anti-doping tests as a consequence of consumption of contaminated dietary supplements. However, this problem is still topical, which is confirmed by regular reports, presented e.g. during the Cologne Workshops on Dope Analysis held annually in Germany. This paper presents a few doping cases caused by the use of supplements with doping substances by athletes, noted by two WADA accredited laboratories (Cologne and Warsaw). Still more education on the health and doping risks of dietary supplement products seems to be necessary for the protection of both athletes and the general public.

KEY WORDS: doping, GC-MS, designer steroid, supplement

INTRODUCTION

The use of nutritional and dietary supplements by athletes is a standard practice in sports at all levels. Several published reports on supplementation in professional sport show high intake of multiple products by some athletes at the same time [1,3,22,28-30]. This occurs although the use of nutritional supplements seems unnecessary when athletes use a well-balanced diet. In addition, many studies do not confirm the ergogenic or anabolic effects of numerous products, which are advertised by their manufacturers as performance enhancing [2,4,8,11,24]. Apart from the biomedical side effects of some supplements used by consumers on their health [10,12], athletes need to be aware that taking these products can cause positive results in doping control [5,6,13]. A failed doping test resulting from the intake of a supplement can originate from the athlete's poor knowledge of banned substances indicated on the label, from the fact that the labelled ingredients indeed contain banned substances or that the supplement contains a banned substance not indicated or concealed on the label [14,15,18,19,21,31]. One should also take into consideration the fact that the list of substances and methods prohibited in sport, published annually by the World Anti-Doping Agency (the WADA Prohibited List), has an open character. In spite of the examples of prohibited substances or methods in particular groups, some additional substances, which are not present on the list, but are characterized by "a similar chemical structure or similar biological effect(s)", can be considered as doping. This was the case for example with 4-methyl-2-hexanamine, which was not indicated on the WADA Prohibited List 2009. However, because of its chemical similarity to the structure of tuaminoheptane, it was recognized by the WADA as a substance banned in sport [23]. In addition, substances specially designed for doping purposes, mainly from the group of anabolic-androgenic steroids, continue to appear on the market. The aim of this study is a warning about the presence of designer steroids in some dietary or nutritional supplements. A few doping cases caused by the use of supplements with designer steroids, noted by two WADA accredited laboratories (Cologne and Warsaw) in recent years, are described.

MATERIALS AND METHODS

Gas chromatography-mass spectrometry (GC-MS) analyses were performed on an Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector with electron ionization (70 eV). The column used was an Agilent Ultra-1 (17 m; 0.20 mm i.d.; 0.1 μm film thickness) using helium as the carrier gas with a constant pressure of 1.14 bar. The oven temperature was increased from 183-232°C by 3°C/min followed by 40°C/min to 310°C and then held for 2 min. Samples were injected in split mode (1:16) at 300°C.

Chemicals and reagents

17β-Hydroxy-17α-methylandrost-4-en-3-one (methyltestosterone) was obtained from Sigma-Aldrich GmbH (Steinheim, Germany) and androsta-1,4,6-triene-3,17-dione and 17β-hydroxyandrosta-1,4,6-trien-3-one from Steraloids (Wilton, USA). 17α-Methyl-5α-androstane-3α,17β-diol (3α,5α-THMT), 17α-methyl-5β-androstane-3α,17β-diol (3α,5β-THMT), 17β-methyl-5α-androstane-3α,17α-diol (3α,5α-epiTHMT), 17β-methyl-5β-androstane-3α,17α-diol (3α,5β-epiTHMT), d_3-testosterone, d_3-epitestosterone, d_4-etiocholanolone, and d_5-androsterone-glucuronide were synthesized in the Cologne doping control laboratory as described by Schänzer et al. [25-27], 17β-hydroxy-17α-methylandrosta-4,6-diene-3-one (Δ6-methyltestosterone), 17α-hydroxy-17β-methylandrosta-4,6-diene-3-one (Δ6-epimethyl testosterone), 3α-hydroxy-6α-methyl-5β-androstan-17-one, 3α-hydroxy-6α-methylandrost-4-en-17-one, 6α-methyl-testosterone, and 3α,17α-dihydroxy-6α-methyl-5β-pregnan-20-one as described by Parr et al. [16, 20]. β-Glucuronidase from *E. coli* was obtained from Roche Diagnostics (Mannheim, Germany), N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other reagents and solvents were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Urine samples

Urine samples from routine doping control, from two WADA accredited laboratories (Cologne and Warsaw), with suspicious results in screening for anabolic androgenic steroids were reanalysed to trace back the administered substance. Two samples suspicious for 17α-methyl-5β-androstane-3α,17β-diol (3α,5β-THMT, samples 1 and 2), three samples for 17β-hydroxy-5β-androst-1-en-3-one (samples 3-5) and two sample with an elevated T/E ratio were included in this investigation (samples 6 and 7). The samples were handled anonymously following the Declaration of Helsinki.

Sample preparation

The samples were prepared according to the routine steroid screening procedure used in the Cologne doping control laboratory [7], in the case of samples 1 and 2 without adding 17α-methyltestosterone as an internal standard: after addition of the internal standard solution (containing d_3-testosterone, d_3-epitestosterone, d_4-etiocholanolone and d_5-androsterone-glucuronide and for samples 3-7 17α-methyltestosterone in addition) 2 mL of urine were incubated at pH 7 with β-glucuronidase from *E. coli* at 50°C for 1 h. The steroids were extracted with 5 mL of TBME at pH 9.6 and the organic layer was evaporated to dryness. The residues were derivatized with trimethyliodosilane (TMIS) reagent (MSTFA/NH_4I/ethanethiol, 1000:2:3, v:w:v) by heating for 20 min at 60°C and 2 μL were injected into the GC-MS. In addition to the standard single ion monitoring (SIM) procedure, the instrument was also operated in SCAN mode. As reference, solutions of the respective steroids in methanol were spiked to blank urine and processed in the same manner.

Supplements analysis

In connection with the cases of samples 1 and 2, the sports federation collected six dietary supplement products from the athletes. They were analysed for the presence of designer steroids by GC-MS in SCAN mode after methanolic extraction and derivatization with TMIS reagent.

RESULTS

The samples initially found suspicious for 3α,5β-THMT (samples 1 and 2) revealed the presence of four steroids with MS fragments of m/z 143. This fragment is recognized as indicative for a 17ξ-methyl-17ξ-hydroxylated D-ring. The extracted ion chromatogram is given in Figure 1. By comparison of the mass spectra and retention times of the compounds with those of reference steroids spiked into blank urine, 17β-hydroxy-17α-methylandrosta-4,6-diene-3-one (Δ6-methyltestosterone), 17α-hydroxy-17β-methylandrosta-4,6-

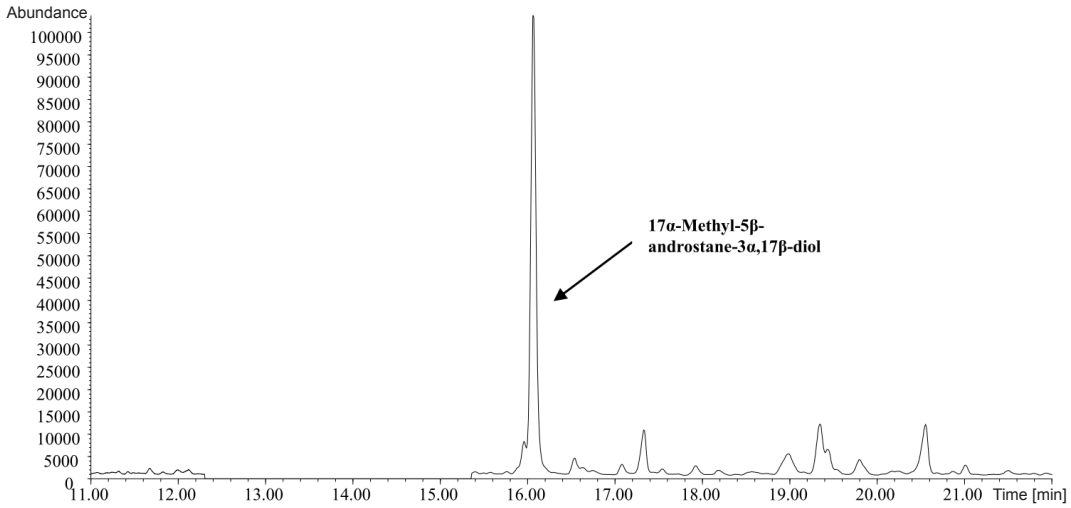

FIG. 1. EXTRACTED ION CHROMATOGRAM (m/z 143) OF SAMPLE 1

diene-3-one (Δ6-epimethyltestosterone), 17α-methyl-5β-androstane-3α,17β-diol (3α,5β-THMT), and 17β-methyl-5β-androstane-3α,17α-diol were identified. Confirmation was performed according to the criteria of WADA [33].

The corresponding supplements were tested for designer steroids and one out of the six products was found to contain Δ6-methyltestosterone. The name of the product label was "Jungle Warfare". The other products did not reveal any steroidal content.

Three samples, initially suspicious for the boldenone metabolite 17β-hydroxy-5β-androst-1-en-3-one (samples 3-5), were found to contain androsta-1,4,6-triene-3,17-dione and 17β-hydroxy-androsta-1,4,6-trien-3-one as major steroids. Additionally, several minor metabolites of androsta-1,4,6-triene-3,17-dione and androst-4-ene-3,6,17-trione were detected as well. A SCAN chromatogram (TIC) of sample 3 is shown in Figure 2.

In sample 6 and 7, initially suspicious due to an elevated T/E ratio, four exogenous steroids, namely 3α-hydroxy-6α-methyl-5β-androstan-17-one, 3α-hydroxy-6α-methylandrost-4-en-17-one, 6α-methyl-testosterone, and 3α,17α-dihydroxy-6α-methyl-5β-pregnan-20-one, were identified besides the altered steroid profile (chromatogram in Figure 3).

DISCUSSION

The reanalysis of samples 1 and 2 resulted in the detection of steroids that may be interpreted as metabolites of Δ6-methyltestosterone. These findings are consistent with the metabolism reported recently by Parr et al. [16]. The availability of the athlete's supplements allowed for confirmation of this interpretation, as one of the products indeed contained Δ6-methyltestosterone. These findings confirmed the presumption that such products are used by athletes and that their consumption may lead to positive results in doping control.

The steroids detected in samples 3-5 most likely trace back to administration of androsta-1,4,6-triene-3,17-dione (ATD). This steroid is reported to inhibit the enzyme complex aromatase. According to our knowledge, no approved pharmaceutical containing ATD is available worldwide. However, it was detected as an active ingredient in supplements such as Novedex XT, Novedex Xtreme, Arom X, Clomed, Estro-Test, Inhibit-E, and ATD [9,17]. In all three urine samples, 3α,6α-dihydroxy-5β-androstan-17-one and other metabolites indicating co-administration of a 6-oxo-steroid were detected as well. Out of the reported ATD products, Novedex XT or Xtreme were the only ones that also contained 3-hydroxy-androst-4-ene-6,17-dione or 6-hydroxyandrost-4-ene-3,17-dione, respectively. Both compounds

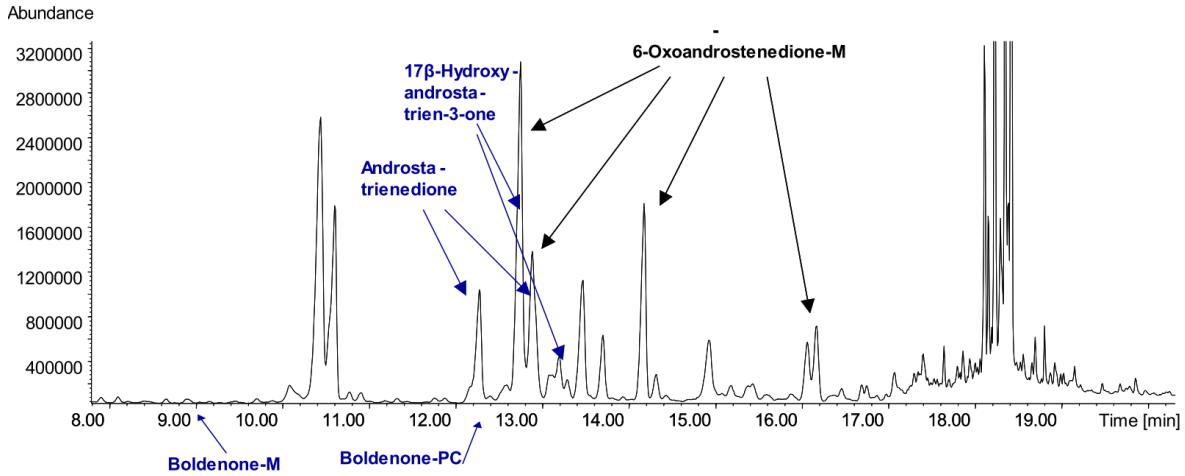

FIG. 2. SCAN CHROMATOGRAM (TIC) OF SAMPLE 3, TESTED POSITIVE FOR METABOLITES OF ANDROSTA-1,4,6-TRIENE-3,17-DIONE (BLUE ARROWS) AND 6-OXOANDROSTENEDIONE (ANDROST-4-ENE-3,6,17-TRIONE, BLACK ARROWS)

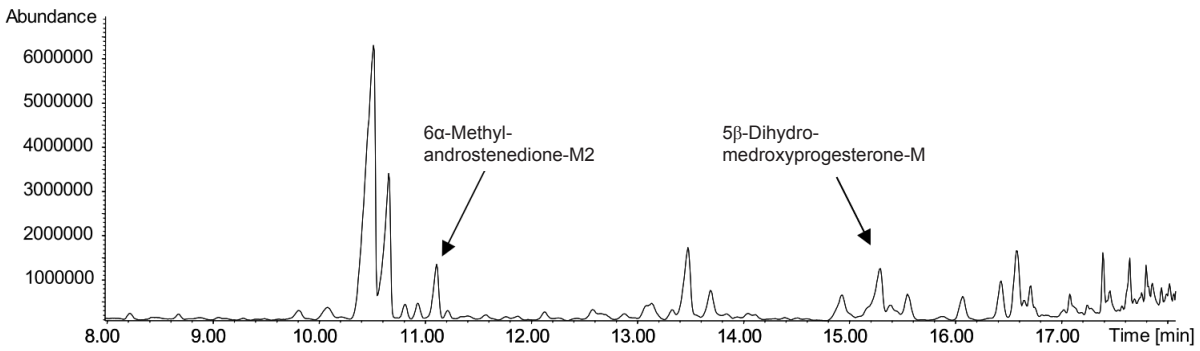

FIG. 3. CHROMATOGRAM OF SAMPLE 6, TESTED POSITIVE FOR METABOLITES OF 6α-METHYLANDROST-4-ENE-3,17-DIONE AND 5β-DIHYDROMEDROXY PROGESTERONE

TABLE I. ADVERSE ANALYTICAL FINDINGS WITH DESIGNER STEROIDS AS REPORTED BY THE WORLD ANTI-DOPING AGENCY 2006-2009 [32].

Designer Steroid	Total	2009	2008	2007	2006
Androsta-1,4,6-triene-3,17-dione	7	1	3	2	1
Androst-4-ene-3,6,17-trione	5	2	2	-	1
Methasterone	6	-	3	3	-
Desoxymethyltestosterone	1	-	1	-	-
6α-Methylandrost-4-ene-3,17-dione	2	-	-	2	-
1-Testosterone and 1-Androstenedione	4	2	-	-	2
Prostanozol	2	-	-	1	1

may be metabolized to 3α,6α-dihydroxy-5β-androstan-17-one. Thus, it is very likely that the athletes in question have administered one of these products.

The findings of metabolites of 6α-methylandrostenedione and 17α-hydroxy-6α-methyl-5β-pregnane-3,20-dione in samples 6 and 7 may be conclusive with the administration of a supplement with those two steroids as active ingredients. This combination was reported for the product "Methyl-1 Pro" [20]. It also contained androst-4-ene-3,17-dione, which might explain the altered urinary steroid profile in the samples.

CONCLUSIONS

From the cases reported here, it is evident that top level athletes use "dietary supplements" that contain so-called designer steroids. The statistics of the World Anti-Doping Agency of recent years reported some more cases (see Table 1) with steroids that are only available in dubious products and not as approved pharmaceuticals. However, people outside of elite sport were also found to have used such designer supplements. Still more education on the health and doping risks of dietary supplement products seems to be necessary for the protection of both athletes and the general public.

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