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Nutritional supplements and doping: Non-labelled multiple prohormones in a Czech nutritional supplement.

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Introduction

Several studies have shown that "non-hormonal" nutritional supplements such as vitamins, creatine, minerals, etc. can be contaminated with anabolic androgenic steroids [1-6]. As a consequence of these findings two ISO-validated methods for the analysis of prohormones were developed in our laboratory. Both methods, one for solid and one for aqueous supplements were previously described [7,8]. Results obtained during the last year with both methods are given in this study.

Positive results can be caused by accidental contamination or by intended adding of prohormones. A case is described of a positive nutritional supplement from Czech origin. respective concentrations could indicate addition of prohormones instead of accidental contamination.

Experimental

Supplement analysis

A distinction was made between solid and aqueous nutritional supplements. Sample preparation for both solid and aqueous nutritional supplements is as previously described [7,8]. Briefly, to 1 g of a solid nutritional supplement 5 ml of 1 N NaOH, 50 µl of the internal standard (androsterone, 2µg/ml, MeOH) and 5 ml of a pentane/diethylether mixture (9:1) are added. After rolling for 1h 1g Na_2SO_4 is added before centrifugation. The organic layer is separated and evaporated under oxygen free nitrogen. 1g of a NaHCO3/K2CO3 buffer is added to 5 ml of an aqueous nutritional supplement together with 50 µl of the internal standard androsterone (2 µg/ml, MeOH) an 5 ml of a pentane/diethylether mixture (1:1). After rolling for 1 h and centrifugation, the organic layer is separated and evaporated under oxygen

free nitrogen. The resulting extracts were derivatised using 100 µl MSTFA/NH_4/ethanethiol (320/1/2) for 1 h at 80°C.

GC/MS analysis was performed using an HP6890 gas chromatograph, equipped with an HP Ultra 1 column (l=17m, i.d.=0.2 mm, d_f=0.11µm) directly coupled to a HP5973 mass selective detector. Detection was performed in the SIM mode. Three diagnostic ions were monitored for each compound [7].

Method validation

The methods were validated according to ISO 17025. Totally 32 compounds were included in this validation procedure. The limit of detection (LOD) was determined using 10 different nutritional supplements for both aqueous and solid nutritional supplements. The LOD was defined as the lowest concentration where a compound could be detected in all 10 spiked supplements using the three ions monitored. Besides the LOD, selectivity (interference of related compounds) and specificity (matrix interferences) were tested as well.

Quantitation of prohormones

The concentration of prohormones present in the Czech supplement (Pyruvate Ca, Czech Republic) was determined on the homogenised content of 10 capsules using an equally weighted linear calibration curve constructed in the range from 0 to 500 ng/g. Therefore a blank nutritional supplement (carbo energizer orange, Performance, Nutrico, Belgium) was spiked with testosterone, dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), 4(5)-androstene-3,17-dione, nandrolone and 19-nor-4(5)-androstene-3,17-dione and analysed at 5 different levels (0, 50, 100, 250 and 500 ng/g) according to the described method. Each level was analysed in triplicate. The quantification ion and qualifier ions for each of these compounds are summarised in Table 1. All calibration curves showed a good linearity (R^2 between 0.977 and 0.990).

Excretion Study

An excretion study using a Pyruvate Ca supplement (Czech Republic) was performed with 3 male volunteers. The purpose of the study was explained to each volunteer.

Each volunteer received 3 capsules at the beginning of the study, followed by three more after 4 h. This was in agreement with the recommendations on the package of the supplement.

A urine sample was taken before the start of the experiment and after the administration the urine was collected quantitatively up to 12 h (0, 1, 2, 4, 6, 9 and 12h).

Results and Discussion

The LOD's of the 32 different compounds range from 2 to 20 ng/g and from 1 to 10 ng/ml for solid and aqueous supplements (Table 2). The tests for selectivity and specificity did result neither in the detection of any interference from related compounds nor from the matrix.

During the period from 12/2002 to 12/2003 theses methods were used for the analysis of 95 nutritional supplements. Of them, 92 supplements were sent to us by nutritional supplement selling companies or suppliers of raw materials. The other three supplements were bought by coworkers of the laboratory. For 13 (13.68%) of these samples no reliable data could be obtained. Most common reasons were matrix interferences such as impures derivatives or oil based supplements.

25 of the 95 samples analysed, or more than a quarter (26,3%), contained prohormones. 17 of those samples contained more than one anabolizing agent. Table 3 summarises the compounds detected during this period and frequence a compound was found. Testosterone and its prohormones were detected most frequentely. Dehydroepiandrosterone (DHEA) was found in almost all positive samples (76%). Other substances commonly detected included 4(5)-androstene-3,17-dione, testosterone, and dihydrotestosterone. Some supplements contained nandrolone and its precursor 19-nor-4(5)-androstene-3,17-dione, but not as frequently as testosterone and/or one of its prohormones.

The results prove that testing supplements for the presence of anabolizing agents results in a large number of positive samples.

Semi quantitative analysis indicates that in some cases the contamination seems to be the result of deliberate addition. One example is the supplement pyruvate Ca from Czech origin. Screening of this nutritional supplement resulted in the detection of 6 compounds (Table 1). The abundance of the monitored ions in this sample exceeded by far those of a quality control sample spiked at 10 ng/g. Confirmation of the screening results could be obtained using full scan mass spectrometry. Figure 1 shows the confirmation results of a diluted sample for dihydrotestosterone in comparison to a quality control sample spiked at 50 ng/g. Analogue results could be obtained for the other compounds.

The concentrations found in this supplement, using the 5-point calibration curve, are shown in Table 4, with the highest amount found for 19-nor-4(5)-androstene-3,17-dione.

Analysis of the urine samples of the excretion study did not result in an elevated T/E ratio nor in changes in the DHT parameters. Analysis for norandrosterone resulted in excretion profiles as shown in Figure 2. All volunteers tested positive according to the WADA threshold level

of 2 ng/ml after the first administration. Some volunteers tested positive up to 9h after the first administration. According to the manufacturer, half of a daily dose (3 capsules) should be taken 1 h before a sport event. Obviously, this may lead to a positive doping test.

The findings by H. Geyer et.al [9] that urinary concentrations of the nandrolone metabolite norandrosterone above the threshold level of 2 ng/ml can be obtained if the total uptake of nandrolone prohormones is higher than 1 μg are confirmed in this excretion study.

Conclusion

High percentages of positive samples for anabolizing agents are reported after one year of analysing nutritional supplements in our laboratory. The substance most frequently detected is DHEA.

An excretion study performed with a contaminated Czech supplement resulted in a positive doping test for norandrosterone.

Athletes should be advised to be very cautious in using nutritional supplements in order to avoid unintended positive doping cases.

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Table 1: Quantification ion (m/z) and qualifier ions used for the quantification of different compounds in nutritional supplements.

compound	Quant. Ion	Qual. 1	Qual. 2
testosterone	432	417	209
DHT	434	405	143
nandrolone	418	403	194
DHEA	432	417	327
19-nor-4(5)-androstene-3,17-dione	416	401	194
4(5)-androstene-3,17-dione	430	415	234

Table 2: LOD's of the 32 compounds for both solid and aqeous nutritional supplements.

compound	LOD Solid (ng/g)	LOD Liquid (ng/ml)	compound	LOD Solid (ng/g)	LOD Liquid (ng/ml)
DHEA	2	1	stanozolol	/	10
1,4-androstadiene-3,17-dione	10	1	metandienone	10	2
19-nor-4-androstene-3,17-dione	2	1	5α-androstane-3β,17β-diol	10	2
5-androstene-3β-17β-diol	5	2	testosterone phenylpropionate	10	2
nandrolone	5	1	testosterone isocaproate	20	/
dihydrotestosterone	5	1	testosterone propionate	5	1
4-androstene-3,17-dione	2	1	testosterone undecanoate	2	1
testosterone	2	1	testosterone decanoate	2	1
19-nor-5-androstene-3,17-dione	2	1	nandrolone decanoate	5	2
5-androstene-3,17-dione	2	1	nandrolone phenylpropionate	5	2
4-androstene-3β-17β-diol	10	1	nandrolone laurate	5	1
5a-androstane-3α,17β-diol	5	1	methyltestosterone	2	1
7-keto-DHEA	/	2	1(5α)-androstene-3,17-dione	5	2
boldenone	10	2	1-testosterone	5	1
4-androstene-19-ol-3,17-dione	/	2	19-nor-5-androstene-3,17-diol	10	1
clenbuterol	10	1	19-nor-4-androstene-3,17-diol	10	1

Table 3: Compounds detected in nutritional supplements during the period 12/2002 – 12/2003 and their occurrence.

Compound	Occurrence
DHEA	19
testosterone	12
4(5)-androstene-dione	9
5-androstene-3β,17β-diol	6
DHT	6
19-nor-4(5)-androstene-dione	5
nandrolone	3
methyltestosterone	2
nandrolone phenylpropionate	1
1-testosterone	1
1-androstene-3,17-dione	1

Table 4: Concentration found (ng/g) and average amount per capsule (ng) detected in the Czech supplement.

Compound	Concentration (ng/g)	Amount/capsule (ng)
DHEA	1047	761
19-nor-4(5)-androstene-3,17-dione	1739	1265
nandrolone	474	345
DHT	218	158
4(5)-androstene-3,17-dione	67	49
testosterone	408	296

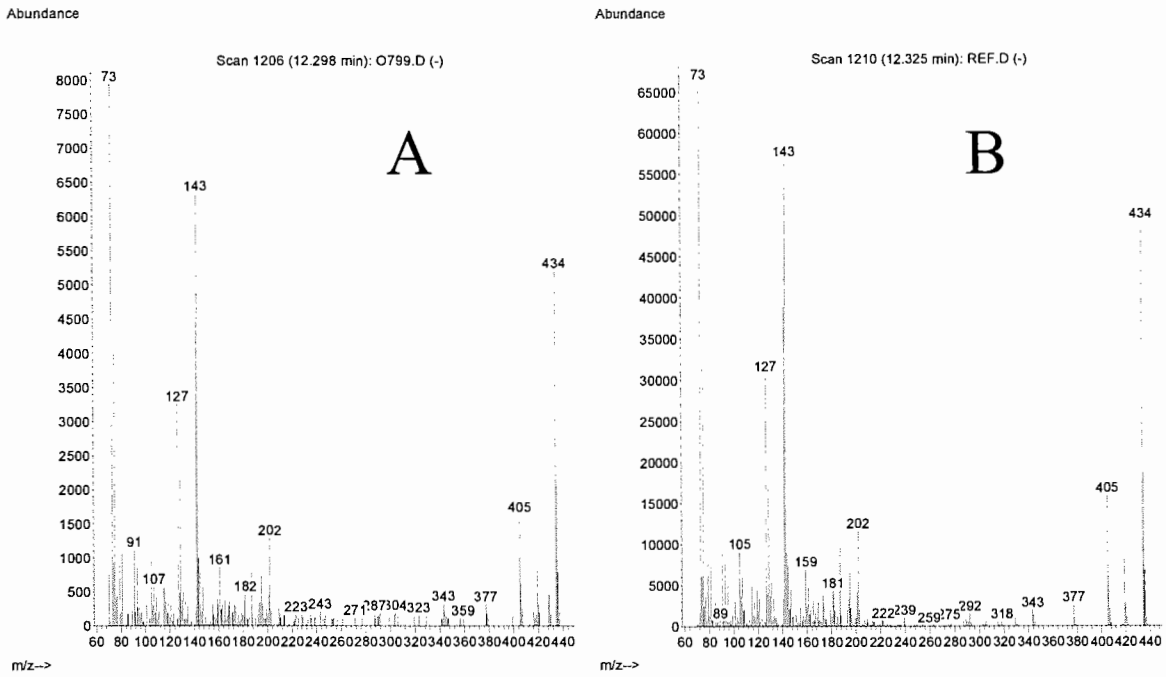

Figure 1: Mass spectrum of TMS-derivatised DHT in a Czech supplement (A) in comparison to a quality control sample (B).

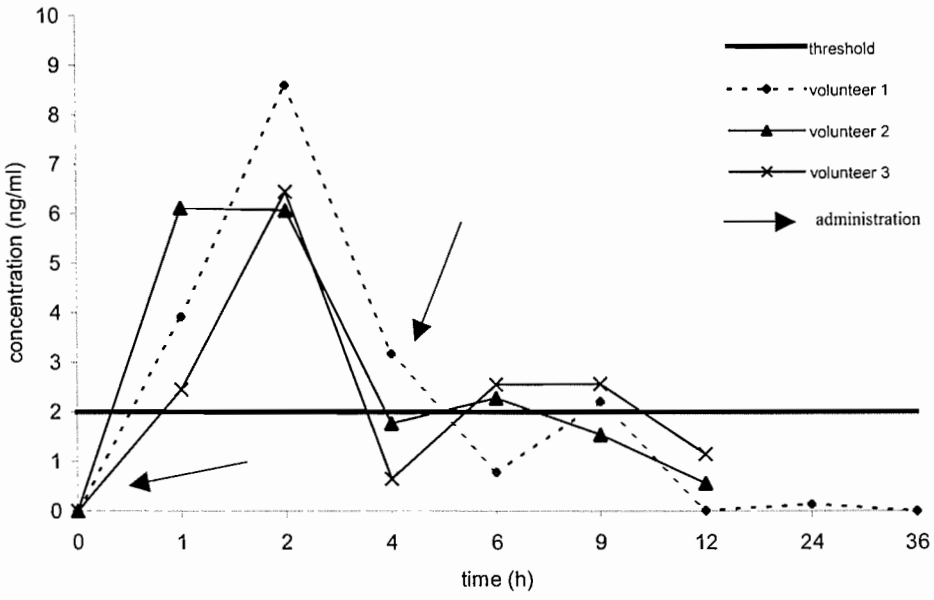

Figure 2: Excretion profiles after the administration of the recommended dose of a Czech nutritional supplement.