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Nutritional supplements and doping: The Ghent Experience

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Introduction

The use of nutritional supplements has exploded at the end of the previous century. For example, the last decade creatine sales numbers have increased from zero to 3.1 million kilograms [1]. The total value of the US supplement market in the year 2000 has been estimated at US$ 16.7 billion [2].

The ever increasing aim for success, stimulated by the high financial stakes in elite sport, and the adoption of the Dietary Supplement Health and Education Act in 1994 [3] has caused a situation where the use of nutritional supplements by athletes has become a matter of concern. Recently, evidence was found that several of these prohormones were present in "non-hormonal" nutritional supplements [4-7]. According to the regulations of the IOC, prohormones belong to the prohibited class of anabolic steroids. In two studies, high doses of the anabolic steroid metandienone were found [8,9]. In both cases, the presence of metandienone was not mentioned on the label.

Since February 2002, 150 nutritional supplements were tested in our laboratory. For 147 samples an analysis for prohormones was requested by the supplier. In 47 cases, an analysis for caffeine was carried out and 70 samples were tested for the presence of ephedrines. Of all samples tested, 18 (12%) contained one or more compounds banned by the IOC doping regulations. 13 (8.84%) supplements, analysed with a full scan method with an LOD of 250 ng/g, contained one or several prohormones not mentioned on the label. 10.6 % of the nutritional supplements contained caffeine.

Of all nutritional supplements tested for anabolic steroids 13.7 % did not give reliable data. The major reasons for this lack of data were that no internal standard could be observed after extraction, no dry residues could be obtained from fish oil based supplements or the derivatisation with the MSTFA-mixture routinely used for the screening of anabolic steroids in urine was not succesfull.

Because of the ever increasing use of nutritional supplements, the detection of several anabolizing agents in nutritional supplements banned by international doping rules and the lack of a standard method according to ISO 17025, validated methods for the screening of anabolizing agents in both aqueous and solid nutritional supplements are presented here.

Experimental

Chemicals and reagents

5α-androstane-3α,17β-diol ; 19-nor-4-androstene-3,17-dione ; 5α-androstane-3β,17β-diol ; 4-androstene-3,17-dione ; boldenone and testosterone were obtained from Sigma (St. Louis, MO, USA). 19-nor-4-androstene-3β,17β-diol ; 19-nor-5-androstene-3,17-dione ; 4-androstene-3β,17β-diol ; metandienone ; 5-androstene-3β,17β-diol ; 5-androstene-3,17-dione ; 4-androstene-19-ol-3,17-dione and 7-keto-dehydroepiandrosterone were bought (7-keto-DHEA) from Steraloids (Newport, USA), dehydroepiandrosterone (DHEA) from Serva (Heidelberg, Germany) and dihydrotestosterone (DHT) from Piette International Laboratories (Drogenbos, Belgium). Nandrolone and stanozolol were bought from NARL (Pymble, Australia). Clenbuterol was obtained from Boehringer Ingelheim (Ingelheim am Rhein, Germany). 17α-methyltestosterone, testosterone propionate, testosterone isocaproate, testosterone decanoate, testosterone phenylpropionate, testosterone undecanoate, nandrolone decanoate and nandrolone phenylpropionate were obtained from Organon (Oss, The Netherlands). Nandrolone laurate, Laurabolin®, was from Intervet International (Boxmeer, The Netherlands). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Chem. Fabrik Karl Bucher (Waldstetten, Germany). All other chemicals were of analytical grade.

GC-MS conditions

The GC-MS analysis was conducted in the SIM mode on an HP 6890 gas chromatograph directly coupled to an HP 5973 mass selective detector (HP, Palo Alto, USA). Three ions were monitored for each compound. The GC column was an HP-Ultra 1 (J&W, Folsom, USA), 100 % methylsilicone column with a length of 17 m, an internal diameter of 0.2 mm and a film thickness of 0.11 µm. Helium was used as the carrier gas (linear velocity: 41 cm/s). A total of 0.5 µl was injected splitless. The oven temperature program was as follows: 120°C (0 min), 70°C/min → 181°C (0 min), 4°C/min → 234°C (0.1 min), 30°C/min → 300 °C (10 min). The electron energy was set at 70 eV and the ion source temperature was set at 230 °C.

Extraction

A distinction was made between aqueous and solid nutritional supplements.

For the aqueous nutritional supplements, 1g of a $NaHCO_3/K_2CO_3$ (2:1) buffer was added to 5 ml of the supplement together with 50 µl of the internal standard 17α-methyltestosterone and 5 ml of a pentane/diethyl ether mixture (1:1). After extraction by rolling for 1h and centrifugation, the organic layer was separated and evaporated under oxygen free nitrogen. The residue was derivatised with 100 µl MSTFA/NH_4I/ethanethiol (320/1/2) for 30 min at 80°C.

Solid nutritional supplements are extracted by adding 5 ml NaOH (1N) to 1 g of supplement. After vortexing very carefully, 50 µl of 17α-methyltestosterone and 5 ml of a pentane/diethyl ether mixture (9:1) are added and extraction performed by rolling for 1 h. Afterwards, 0.5-1.0 g of anhydrous Na_2SO_4 is added. After centrifugation the organic layer is separated and dried under oxygen free nitrogen. Derivatisation is as above.

Method validation

The analytical method validation for the screening of 27 compounds was performed according to ISO 17025 on ten different nutritional supplements, randomly chosen, for both aqueous and solid supplements.

To determine the LOD's, the different nutritional supplements were spiked with a reference mixture at different concentrations in the range 1 to 80 ng.

Selectivity was tested by the analysis of a reference miture of 10 structurally related compounds at a concentration of 200 ng/g. These compounds were: 19-noretiocholanolone, 17α-trenbolone, oxymesterone, 3'-OH-stanozolol, mesterolone, salbutamol, terbutaline, etiocholanolone, 5β-andrest-1-ene-17β-ol-3-one and oxandrolone. Matrix interferences were tested by the analysis of the 20 different nutritional supplements.

Results and discussion

Totally 27 analytes were screened for (Table 1) including prohormones of nandrolone and testosterone. Additionally, esters of both compounds were included in the method.

Under the chromatographic conditions described, the internal standard gave a sharp peak with a retention time of 14.55 min. The GC relative retention times and monitored ions (3 per compound) are summarised in Table 1.

No matrix interferences were found at the retention times of the 27 compounds and the

internal standard. No interferences were detected during the analysis of the 10 structurally related compounds. Hence, this method seems to be specific and selective.

The LOD was defined as the concentration whereby a compound could be detected with a certainty of 100 % in case of ten spiked supplements. The resulting LOD's of the 27 compounds in both aqueous and solid nutritional supplements are given in Table 2. Generally, the LOD's for aqueous nutritional supplements are lower than for solid nutritional. The most obvious reason is that the matrix of solid nutritional is more complex. As can be seen, eleven analytes have an LOD of 1 ng/ml, or the lowest spiked concentration for the aqueous nutritional supplements. For the liquid supplements, all compounds can be detected at or below 10 ng/ml. For the more complex solid nutritional supplements, the highest LOD is 40 ng/g.

Several supplements previously analysed in the full scan mode and declared negative were reanalysed with this new SIM method. For two of them, one aqueous and one solid nutritional supplement, following results were noticed.

The first case was a creatine serum. Previous analysis of this nutritional supplement in the full scan mode did not result in the detection of unauthorised compounds. Therefore, this nutritional supplement was used as a negative matrix in the validation process of the aqueous nutritional supplements. Surprisingly, the test for specificity resulted in the detection of DHEA in very low concentrations. Confirmation of this screening result could be performed by GC-MS^2. The resulting daughter spectrum of DHEA in the creatine serum in comparison with a quality control sample is given in Figure 1.

More surprisingly, 6 forbidden substances were found in a solid nutritional supplement previously declared negative with the full scan screening procedure. These substances were: DHEA, DHT, testosterone, 4(5)-androstene-dion, 5-androstene-3β,17β-diol and 19-nor-4(5)-androstene-3,17-dion. The presence of each of these compounds, excepting DHT, was confirmed with GC-MS^2. As an example, the daughter spectrum of 5-androstene-3β,17β-diol is shown in Figure 2.

In conclusion, a reliable and sensitive method has been validated for the screening of prohormones in nutritional supplements. 27 analytes could be detected in solid as well as aqueous nutritional supplements. The limit of detection ranged between 1 and 10 ng/ml for the aqueous nutritional supplements and between 2 and 40 ng/g for the solid ones.

Following international doping regulations, athletes remain responsible for the presence of doping substances in their biofluids. Because several studies have shown that nutritional supplements can be contaminated with anabolizing agents, athletes should be very cautious

using nutritional supplements. The development of a sensitive and ISO 17025 validated screening method for the detection of anabolizing agents in nutritional supplements could be helpful for manufacturers to avoid unintended contamination of their products.

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Table 1: Relative retention times (RRT) and monitored ions (m/z) for trimethylsililated compounds.

Compound	RRT	monitored ions (m/z)
clenbuterol	0.34	335.1 , 300.1 , 86.1
5α-androstane-3α,17β-diol	0.77	436.4 , 331.2 , 241.2
19-nor-4-androstene-3,17-diol	0.78	420.3 , 330.2 , 240.2
DHEA	0.82	432.3 , 417.3 , 327.2
19-nor-(4)5-androstene-3,17-dione	0.83	416.3 , 401.2 , 194.1
4-androstene-3β-17β-diol	0.83	434.3 , 405.3 , 143.1
5-androstene-3β-17β-diol	0.85	434.3 , 344.3 , 239.2
5α-androstane-3β,17β-diol	0.85	436.4 , 421.3 , 241.2
nandrolone	0.85	418.3 , 403.3 , 194.1
DHT	0.87	434.3 , 405.3 , 143.1
4(5)-androstene-3,17-dione	0.88	430.3 , 415.3 , 234.1
boldenone	0.89	430.3 , 415.3 , 206.1
testosterone	0.90	432.3 , 417.3 , 209.0
metandienone	0.98	444.3 , 339.2 , 206.1
methyltestosteron	1.00	446.3 , 356.2 , 301.2
4-androstene-19-ol-3,17-dione	1.01	518.4 , 428.3 , 415.3
7-keto-DHEA	1.03	518.3 , 429.2 , 296.1
testosterone propionate	1.05	416.3 , 401.3 , 343.2
stanozolol	1.15	472.4 , 457.3 , 143.1
testosterone isocaproate	1.16	458.4 , 443.3 , 343.2
nandrolone decanoate	1.32	500.4 , 485.4 , 329.2
nandrolone phenylpropionate	1.33	478.3 , 463.3 , 194.1
testosterone decanoate	1.35	514.4 , 499.4 , 343.2
testosterone phenylpropionate	1.36	492.4 , 477.3 , 105.0
testosterone undecanoate	1.41	528.5 , 513.4 , 343.2
nandrolone laurate	1.45	528.5 , 513.4 , 329.2

Table 2: LOD's of the 27 compounds screened for in both aqueous and solid nutritional supplements.

Compound	LOD liquid (ng/ml)	LOD solid (ng/g)
DHEA	1	2
19-nor-4(5)-androstene-3,17-dione	1	2
nandrolone	1	5
4(5)-androstene-3,17-dione	1	2
4-androstene-3β-17β-diol	1	5
4-androstene-19-ol-3,17-dione	1	40
testosterone undecanoate	1	5
testosterone decanoate	1	5
nandrolone phenylpropionate	1	5
nandrolone laurate	1	5
testosterone propionate	1	2
metandienone	2	5
19-nor-4-androstene-3,17-diol	2	2
5-androstene-3β-17β-diol	5	10
DHT	5	5
testosterone	2	2
5α-androstane-3α,17β-diol	5	5
7-keto-DHEA	5	40
boldenone	5	10
clenbuterol	5	5
5α-androstane-3β,17β-diol	5	5
testosterone phenylpropionate	5	20
testosterone isocaproate	5	20
nandrolone decanoate	5	10
stanozolol	10	40

Figure 1: Daughter spectrum of DHEA (precursor m/z = 432) in a creatin serum.

A: Creatin serum.

B: quality control sample.

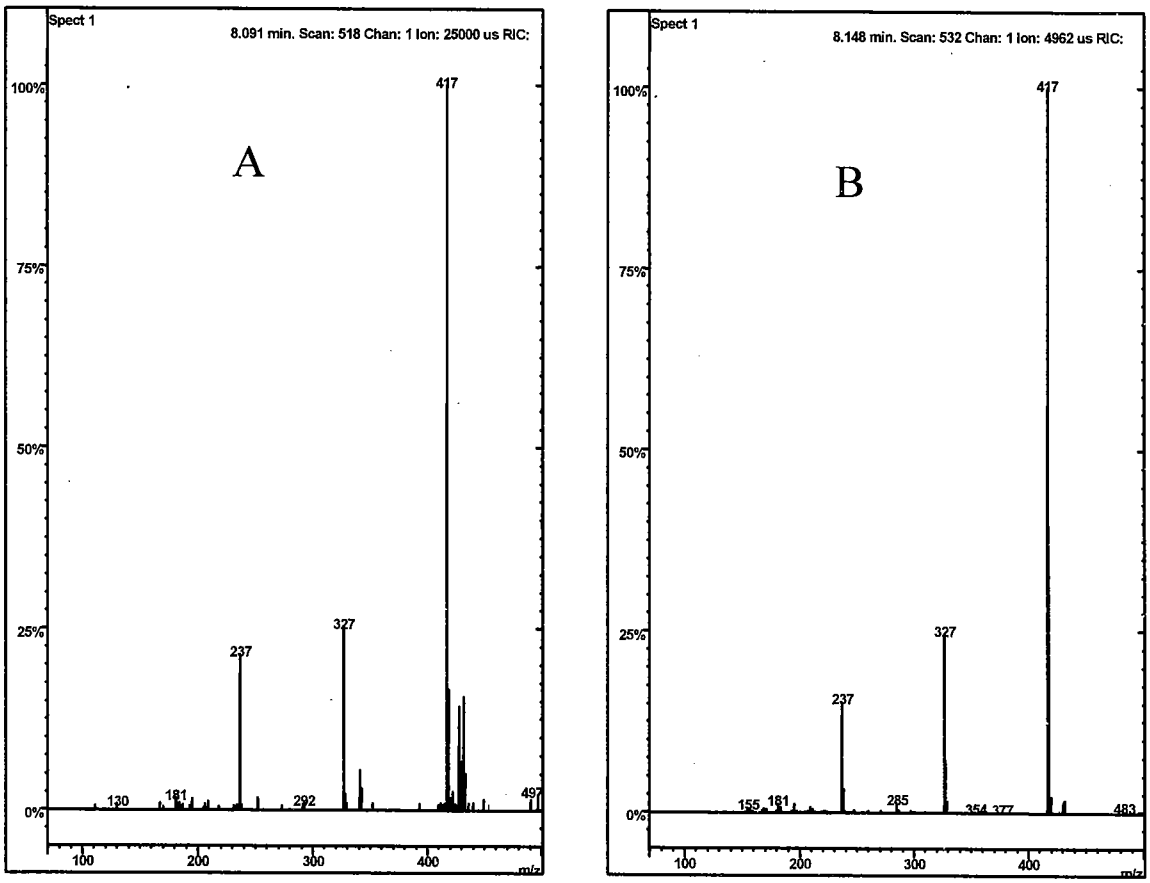
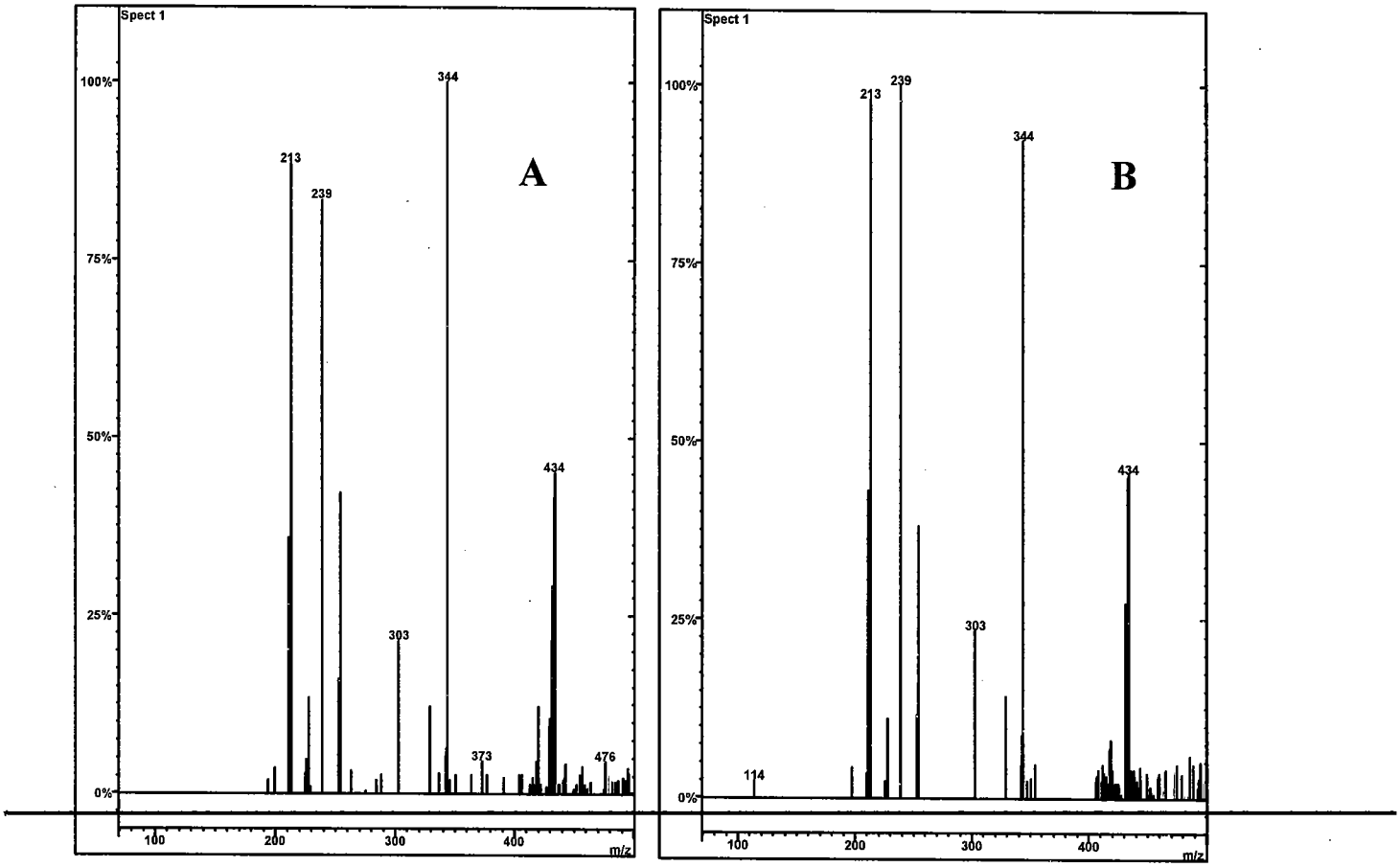


Figure 2: Daughter spectrum (precursor m/z=434) of 5-androstene-3β,17β-diol in a solid nutritional supplement.

A: Nutritional supplement X.

B: 5-androstene-3β,17β-diol.



References:

[1] Maughan R. The scope of nutritional supplement use in sport. In: Schänzer W. Delbeke F.T., Delogiannis A., Gmeiner G., Maughan R. and Mester J, Health and doping risks of nutritional supplements and social drugs 2002.

[2] Finacial Times, April 19th 2002.

[3] Dietary Supplement Health and Education Act of 1994. Pub L No. 103-417, 1994. 103rd Congress, 2nd sess, S784.

[4] Geyer H, Mareck-Engelke U, Reinhart U, Thevis M and Schänzer W. Positive dopingfälle mit norandrosteron durch verunreinigte nahrungsergänzungsmittel. *Dtsch. Z. Sportmed.* 2000, **51**, **11** 378.

[5] Geyer H, Mareck-Engelke U, Wagner A,and Schänzer W. Analysis of "non-hormonal" nutritional supplements for prohormones. In: Schänzer W, Gotzmann A, Mareck-Engelke U, Recent advances in doping analysis. Sport und Buch Strauβ, Koln 2001 **9** 63.

[6] De Cock KJS, Delbeke FT, Van Eenoo P, Desmet N, Roels K and De Backer P. Detection and determination of anabolic steroids in nutritional supplements. *J Pharm Biomed Anal.* 2001 **25** 843.

[7] Geyer H, Parr MK, Mareck U, Reinhart U, Schrader Y and Schänzer W. Doping Substances in Nutritional Supplements, Results of the International IOC Study: Analysis of Non-Hormonal Nutritional Supplements for Anabolic Androgenic Steroids. in: Schänzer W, Delbeke F, Delogiannis A, Gmeiner G, Maughan R, Mester J, Health and doping risks of nutritional supplements and social drugs 2002.

[8] Geyer H, Bredehöft M, Mareck U, Parr MK and Schänzer W. Hoge dosen des anabolikums metandienon in nahrungsergänzungsmittel. *Dtsch. Apoth. Ztg.* 2002b **142**, **29** 50.

[9] Gmeiner G. Metandienon in sportnahrung. *Oster. J. Sportmed.* 2002 **2** 33.