Analysis of Non-Hormonal Nutritional Supplements for Anabolic-Androgenic Steroids – Results of an International Study

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Abstract

Several recent studies have shown evidence of some nutritional supplements containing prohibited anabolic androgenic steroids, so-called prohormones, which were not declared on the label. Therefore, a broad-based investigation of the international nutritional supplement market was initiated to clarify the extent of this problem. From October 2000 until November 2001, 634 non-hormonal nutritional supplements were purchased in 13 countries from 215 different suppliers. Most supplements were bought in shops in the respective countries (578 samples = 91.2%) and on the internet (52 samples = 8.2%). 289 supplements were from prohormone-selling companies and 345 supplements came from companies which do not offer prohormones. After isolation from the supplement matrix 11 different anabolic androgenic steroids, mainly prohormones of testosterone and nandrolone, were analysed by gas-chromatography/mass spectrometry. Out of the 634 samples analysed 94 (14.8%) contained anabolic androgenic steroids not declared on the label ("positive supplements"). We could not obtain reliable data for 66 samples (10.4%) due to matrix effects. In relation to the total number of products purchased per country, most of the positive supplements were bought in the Netherlands (25.8%), in Austria (22.7%), in the UK (18.8%) and the USA (18.8%). According to the label, all positive supplements were from companies located in only five countries: the USA, the Netherlands, the UK, Italy and Germany. 21.1% of the nutritional supplements from prohormone-selling companies contained anabolic androgenic steroids, whereas 9.6% of the supplements from companies not selling prohormones were positive. The positive supplements showed anabolic androgenic steroid concentrations of 0.01 µg/g up to 190 µg/g. The administration of supplements containing nandrolone prohormones adding up to a total uptake of more than 1 µg resulted in positive doping results for norandrosterone for several hours.

Key words
Nutritional supplements · doping · prohormones · anabolic-androgenic steroids

Introduction

Since 1996 anabolic androgenic steroids, so-called prohormones, are available on the US sports nutrition market. These substances are advertised as having enormous properties to increase muscle growth and strength. According to the doping regulations of the IOC, these substances belong to the prohibited class of anabolic agents [9]. Several studies have shown that the labelling of prohormone supplements does not reflect their actual content. Many prohormone products contain prohormones as well as concentrations different from those declared on the labels [1,2,8,10,13]. These mislabelling problems indicate an insufficient surveillance and quality control of the production of dietary supplements, especially with respect to the prohormone industry.

Based on this knowledge, non-hormonal dietary supplements such as vitamins, minerals, and amino acids have also been analysed for prohormones. In several recent follow-up studies of positive doping cases it could be shown that even non-hormonal nutritional supplements may contain prohormones not declared

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on the label [5, 6]. These prohormones may lead to positive results in doping tests, especially for the nandrolone metabolite norandrosterone. To clarify to which extent nutritional supplements contain not-declared prohormones, a broad-based investigation of the international nutritional supplement market was conducted.

Materials and Methods

Supplement description

From October 2000 until November 2001, 634 non-hormonal nutritional supplements were purchased in 13 different countries (Table **3**). They were bought in shops (578 samples = 91.2%), on the internet (52 samples = 8.2%) and by telephone order (2 samples = 0.3%). Two samples (0.3%) were sent by the IOC.

The supplements were from 215 companies located in 15 different countries (Table **1**). To our knowledge 57 of those companies also sell prohormones. 289 of the supplements (45.6%) were purchased from prohormone-selling companies, 345 supplements (54.4%) from companies not selling prohormones. In terms of the formulations of the supplements we analysed 316 capsules, 231 tablets, 72 powders and 15 fluids.

Table **1** Origin of the nutritional supplements (according to the label)

Country	*No. of companies*	*No. of samples*
USA	105	408
Germany	31	59
UK	5	37
Netherlands	12	27
Italy	15	22
France	13	19
Switzerland	6	14
Norway	7	12
Belgium	8	10
Spain	5	7
Denmark	4	6
Sweden	1	6
Austria	1	5
Finland	1	1
Portugal	1	1
total	215	634

Analysis of the nutritional supplements

The analysis was performed according to the procedure described by Geyer et al. [5] with certain modifications. At least 10 pulverised tablets or the content of at least 10 capsules were homogenised. 1 g of the homogenised supplement is dissolved in 5 ml methanol. 20 µl of an internal standard solution (mixture of d3-nortestosterone, d3-testosterone and d3-noretiocholanolone in methanol 2.5 µg/ml each) is added. After shaking for 10 min and centrifugation for 5 min at 3000 rpm the methanolic layer is transferred to another test tube and evaporated to dryness with a vacuum rotating evaporator. To the dried residue 5 ml of a sodium hydroxide solution 0.1 M (the pH is adjusted to values above 11.5) and 5 ml of n-pentane are added. After shaking for 5 min and centrifugation the n-pentane layer is transferred to another test tube. 2 ml of a methanol/water solution (95 : 5, v : v) are added to the n-pentane, shaken for 5 min and centrifuged 5 min at 3000 rpm. The n-pentane layer is discarded and 2 µl of a solution of 1-(N,N-diisopropylamine)-alkanes in methanol (Dipa 14 – 23, 0.5 µg/ml each) are added. The methanolic solution is evaporated to dryness in vacuo. The dry residue is derivatised with 100 µl of N-methyl-N-trimethylsilyltrifuoracetamide/NH_4I/Ethanthiol (1000 : 2 : 3 v : w : v) at 60 °C for 20 min. 3 µl are injected into the gas chromatograph coupled to a mass selective detector (HP 6890/HP 5973) and analysed in the SIM-mode with the characteristic ions for the bis-TMS derivatives of the steroids of interest. For the gas-chromatographic separation a cross-linked capillary OV1 column (HP 5 MS column, 16.5 m × 0.2 mm i.d., film thickness 0.25 µm) is used. The oven temperature was programmed from 100 °C at 40 °C/min to 190 °C at 5 °C/min to 240 °C and at 40 °C/min to 320 °C.

The following anabolic-androgenic steroids were included in the screening: testosterone and prohormones of testosterone: 4-androstene-3,17-dione (4-Andendion), dehydroepiandrosterone (DHEA), 5-androstene-3β0,17β-diol (5-Andendiol), 4-androstene-3β0,17β-diol (4-Andendiol), and testosterone (Test); nandrolone (19-nortestosterone) and prohormones of nandrolone: 19-nor-4-androstene-3,17-dione (4-Norendion), 19-nor-4-androstene-3β0,17β-diol (4-Norendiol), 19-nor-5-androstene-3β0,17β-diol (5-Norendiol) and 19-nortestosterone (Nortest); the prohormone of boldenone: androstadiendion (Adiendione) and the prohormone of 5α-dihydrotestosterone: 5α-androstane-3β0,17β-diol (5 a – Adiol).

For confirmation of the presence of the TMS derivatives of 4-androstene-3,17-dione (4-Andendion), 19-nor-4-androstene-3,17-dione (4-Norendion), testosterone (Test) and 19-nortestosterone (Nortest) GC/MS/MS was used additionally to receive more diagnostic ions. The following conditions were applied: GC/MS/MS system: GC Finnigan, GCQ; Injection: 2 µl; at 325 °C; column: HP Ultra 1, 14 m, 0.25 mm i.d., 0.11 µm film thickness; carrier gas: Helium, split 1 : 10, head pressure 10 psi; temperature programme: 100 °C with 40 °C/min to 190 °C, with 5 °C/min to 240 °C, with 40 °C/min to 320 °C, 3 min hold time; ionisation: 70 eV, electron impact (EI).

For identification and quantification the following reference standards were used: DHEA, testosterone, 5α-androstane-3β0,17β-diol and 19-nor-4-androstene-3,17-dione from Sigma, St Louis, USA; 5-androstene-3β0,17β-diol and 19-nortestosterone from Serva, Heidelberg, Germany; 4-androstene-3β0,17β-diol, 19-nor-5-androstene-3β0,17β-diol and androstadiendione from Steraloids, Wilton, USA; 4-androstene-3,17-dione from Schering, Berlin, Germany. The reference standard 19-nor-4-androstene-3β0,17β-diol was synthesized in our laboratory. As internal standard were used d3 – 19-noretiocholanolone from Steraloids, Wilton, USA, and d3-testosterone and d3-nortestosterone, which were synthesized in our laboratory.

The limit of detection of the method was evaluated in different matrices for all substances mentioned above, and was estimated at about 0.005 – 0.1 µg/g. Only concentrations above 0.01 µg/g were considered in this study.

The recoveries were determined in different matrices and are listed in the following for a creatine matrix: 5-Norendiol 82%; 4-Norendiol 70%; DHEA 55%; 4-Andendiol 81%; 4-Norendion 33%; 5-Andendiol 92%; Nordest 32%; 4-Andendion 43%; Test 54%.

Excretion studies with nutritional supplements

Four male volunteers (age: 42.7 ± 6.8 years, height: 173.0 ± 3.4 cm, weight: 70.5 ± 3.4 kg) received several nutritional supplements with known prohormone concentrations. The volunteers were users of the administered supplements. They were informed about the content of low amounts of prohormones in the products and they gave a written consent to participate in an excretion study. Before the administration of the products it could be shown that their urine did not contain norandrosterone and no testosterone/epitestosterone ratio higher than six. Before and several hours after the administration of the supplements, urine samples were collected and analysed with gas chromatography/mass spectrometry for the glucuronides of norandrosterone, testosterone and epitestosterone, according to the screening procedure for anabolic steroids [3].

Results

Nutritional supplements

Out of the 634 samples analysed 94 (14.8%) samples contained prohormones not declared on the label. We could not obtain reliable data for 66 samples (10.4%) because of matrix effects. Out of all positive supplements 23 samples (24.5%) contained prohormones of nandrolone and testosterone, 64 samples (68.1%) only contained prohormones of testosterone, 7 samples (7.5%) only contained prohormones of nandrolone. None of the samples contained the prohormone of boldenone.

Most of the positive results were found for capsules. 19.6% of the capsules (62 of 316), 11.7% of the tablets (27 of 231) and 6.9% of the powders (5 of 72) showed positive results. In the 15 analysed fluids no anabolic steroids were found. The main ingredients declared on the labels of positive samples are listed in Table **2**. Anabolic androgenic steroids were found in nearly all kinds of nutritional supplements.

The distribution of the concentrations of anabolic androgenic steroids in the positive nutritional supplements is shown in Figs. **1 – 3**. 24.5% of the positive supplements showed a total anabolic androgenic steroid concentration higher than 5 µg/g. Four samples (4.3%) had a testosterone concentration higher than 400 ng/g. With regard to the prohormones of nandrolone, 12.8% of the positive supplements contained more than 1 µg/g (Fig. **2**).

The number of prohormones per positive sample is presented in Fig. **4**. In 47.9% of the positive samples more than 1 prohormone was found. 2 samples (2.1%) contained as many as 7 different prohormones.

Table **2** Main ingredients declared on the labels of positive samples

amino acids/(hydrolysed) proteins	*lysophosphatidylcholin*
minerals	*methylsulfonylmethane*
herbal extracts	*enzymes*
vitamins	*(N-acetyl) glucosamine (sulfate)*
carnitin/acetyl-L-carnitine	*chrysin*
creatine	*melatonin*
HMB	*hemoglobin*
pyruvate	*inosine*
ribose	*NAC*
fatty acids/oils	*inositol*
caffeine	*lecithin*
chondroitin (sulfate)	*choline*

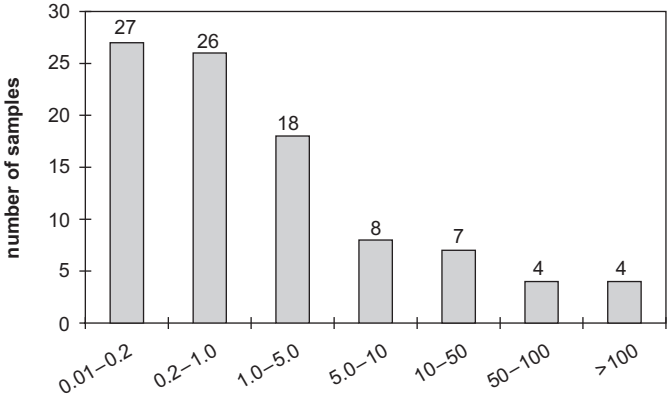

Fig. **1** Distribution of the total prohormone concentrations (µg/g) in positive samples (n = 94).

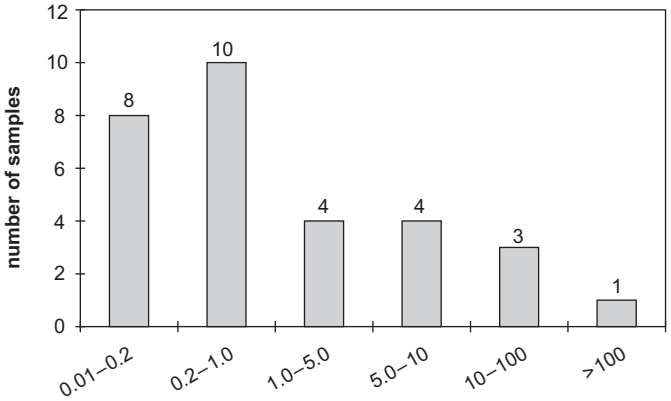

Fig. **2** Distribution of the nandrolone prohormone concentrations (µg/g) in positive samples (n = 94).

Most positive samples contained DHEA (63.2%), 4-Andendion (46.3%), 4-Norendion (27.4%), 4-Andendiol (27.4%) and/or 5-Andendiol (21.1%) (Fig. **5**).

Most of the positive products were bought in the USA and in Germany. In relation to the total number of products purchased, most positive supplements were bought in the Netherlands (25.8%), in Austria (22.7%), the UK (18.9%) and the USA (18.8%, Table **3**).

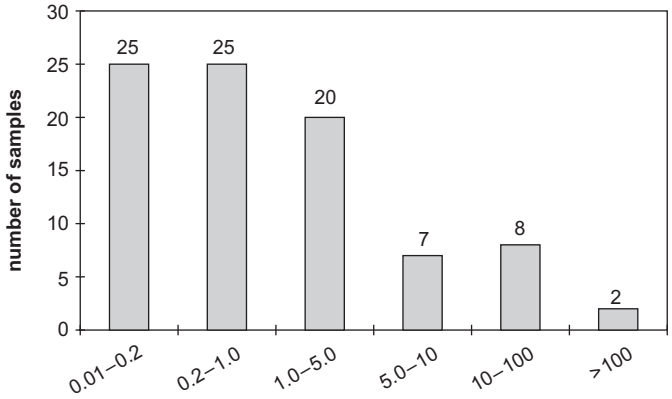

Fig. **3** Distribution of the testosterone prohormone concentrations (µg/g) in positive samples (n = 94).

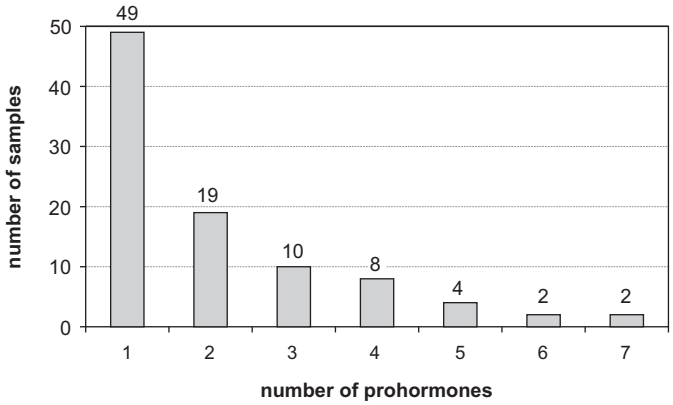

Fig. **4** Number of detected steroids per positive supplement.

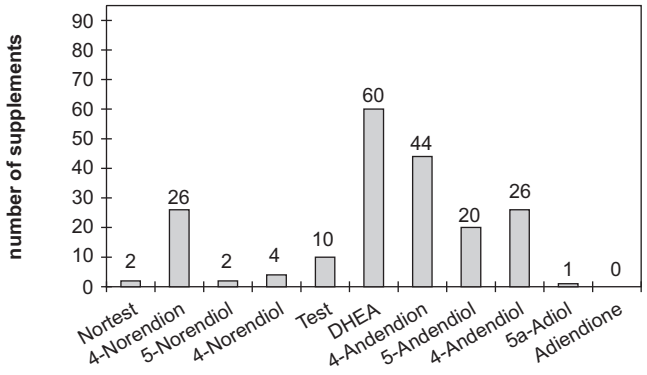

Fig. **5** Number of positive supplements containing specific prohormones.

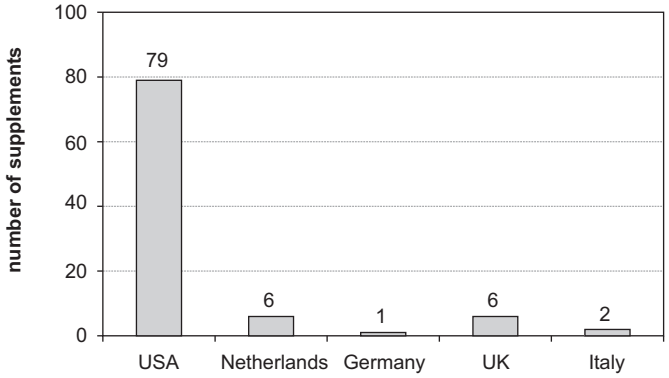

Fig. **6** Origin of the positive samples according to the label.

According to the label all positive supplements were from companies located in only five countries: the USA, the Netherlands, the UK, Italy and Germany (Fig. **6**).

21.1% of the nutritional supplements from prohormone-selling companies contained anabolic androgenic steroids, whereas 9.6% of the supplements from companies not selling prohormones were positive (Fig. **7**).

Excretion studies

Excretion studies with supplements which contained 19-nor-4-androstene-3,17-dione (4-Norendion) in amounts of 0.8 µg, 3.6 µg, 5.6 µg and 24.6 µg resulted in maximum urinary norandrosterone concentrations of 0.5 ng/ml, 11.6 ng/ml, 12.6 ng/ml and 18.0 ng/ml, respectively, after 2 hours (Fig. **8**).

After the application of various small amounts of prohormones of testosterone the ratio of testosterone/epitestosterone showed the normal variation and did not increase.

Discussion

The results of this broad-based study, showing that about 15% of the analysed non-hormonal supplements contained undeclared anabolic androgenic steroids, confirm the results of preliminary studies [5, 6].

Table **3** Nutritional supplements containing prohormones, in relation to the total number of supplements purchased in different countries

Country	*No. of products*	*No. of positives*	*Percentage of positives*
Netherlands	31	8	25.8%
Austria	22	5	22.7%
UK	37	7	18.9%
USA	240	45	18.8%
Italy	35	5	14.3%
Spain	29	4	13.8%
Germany	129	15	11.6%
Belgium	30	2	6.7%
France	30	2	6.7%
Norway	30	1	3.3%
Switzerland	13	–	–
Sweden	6	–	–
Hungary	2	–	–
total	634	94	14.8%

In the present study, about 32% of all positive supplements contained prohormones of nandrolone. This proportion was much higher (83%) in a previous study conducted in our laboratory

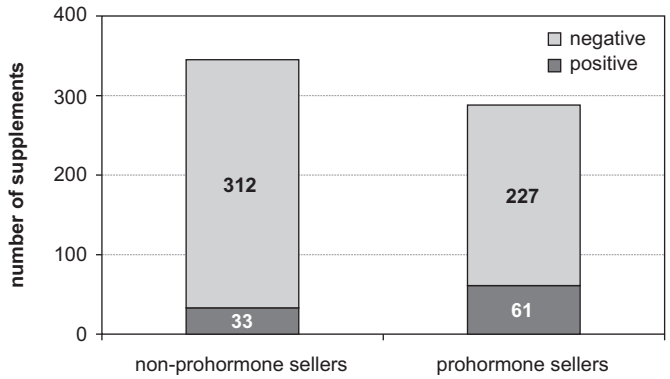

Fig. **7** Number of positive supplements among products from prohormone and non-prohormone sellers.

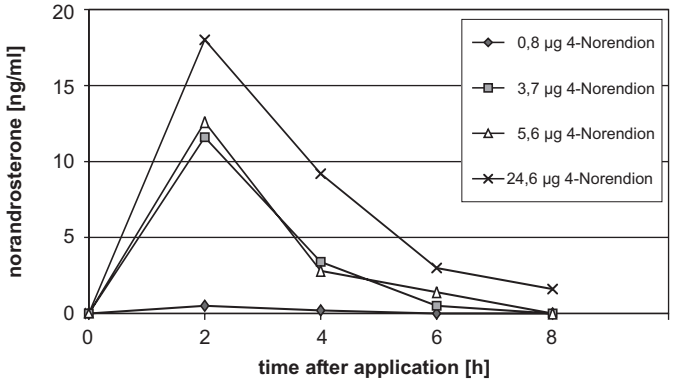

Fig. **8** Urinary concentration of norandrosterone after the administration of nutritional supplements containing different amounts of 19-nor-4-androstene-3,17-dione (4-Norendion).

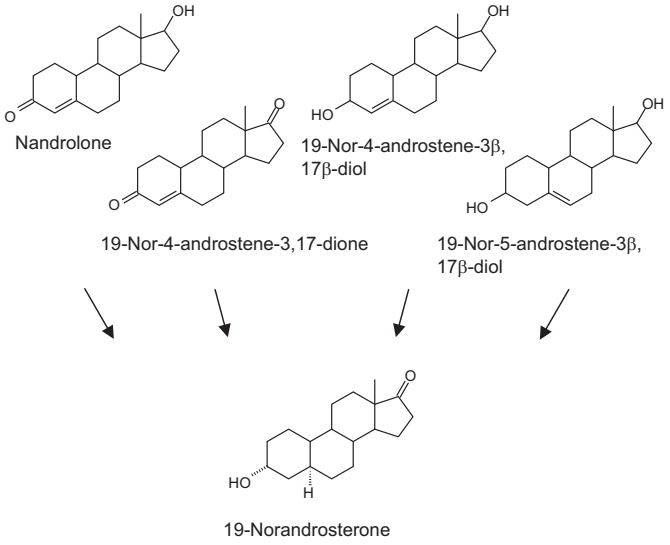

Fig. **9** The urinary norandrosterone as a common metabolite of nandrolone (19-nortestosterone) and nandrolone prohormones.

[6]. This difference is probably due to the fact that previously investigated nutritional supplements were mainly analysed in follow-up studies of positive norandrosterone cases. For about 10% of the analysed nutritional supplements we could not obtain reliable data, due to analytical problems with the matrix. Changes of the analytical procedures, especially in the extraction and derivatisation steps, could not improve the results. Momentarily we do not exactly know what kind of matrix is responsible for the analytical problems.

The concentrations of the anabolic androgenic steroids in the nutritional supplements are rough estimates. In some matrices and for some anabolic-androgenic steroids, especially DHEA, concentrations lower 0.01 µg/g could be detected. These results were not reported. The amounts of anabolic androgenic steroids in the analysed supplements are much lower than the amounts in the lowest concentrations found in commercially available prohormone supplements. These contain at least 10 000 µg of prohormones (Figs. **1 – 3**).

According to studies with androstendione [4,11,12], physiological effects or adverse effects after the oral administration of amounts as low as those detected in the positive nutritional supplements, are unlikely but cannot be entirely excluded.

We suppose that the low concentrations of anabolic androgenic steroids in non-hormonal nutritional supplements are not intentional admixtures to improve the claimed "performance enhancing effects" of a supplement, but are so-called cross-contaminations. These cross-contaminations are possible, because manufacturers of nutritional supplements do not have to adhere to GMP (Good Manufacturing Practices), as required for the pharmaceutical industry. We suppose that contaminations occur in companies which process and transport prohormones. If the same machines and vessels are used for the processing of other nutritional supplements such as vitamins, minerals, etc., contaminations of these "non-hormonal" supplements could result from insufficient cleaning of the processing tools and vessels. Another indicator of cross-contamination is the varying concentration of contaminants found in different products. Such varying concentrations have also been shown in our previous studies [5,6].

In most positive products DHEA, 4-Andendion, 4-Andendiol and 4-Norendion were found (Fig. **5**). These anabolic androgenic steroids are probably the most common prohormones on the nutritional supplement market. Testosterone was found in 10.6% of the positive nutritional supplements. Testosterone is not permitted in the nutritional supplement market. Testosterone is probably a by-product of the synthesis of testosterone prohormones, which is not sufficiently separated after synthesis. In previous studies on prohormones testosterone has also been detected in prohormone supplements [1].

In all countries except Switzerland, Sweden and Hungary positive nutritional supplements were among those purchased (Table **3**). However, fewer supplements were bought in those countries. In relation to the total number of supplements purchased in different countries, most positive supplements were bought in the Netherlands (25.8%), Austria (22.7%), the UK (18.9%) and the USA (18.8%) (Table **3**). The high percentage of positive nutritional supplements from Austria is confirmed in a recently published paper. In this study the analysis of about 50 nutritional supplements found that 20% of the supplements contained anabolic-androgenic steroids not declared on the label [7].

As shown in Fig. **6**, most positive supplements are from companies which, according to the label, are located in the USA. In most cases the label does not clearly indicate where a nutritional supplement has been produced. According to the legislation it is not necessary for nutritional supplement companies to list this information on the label. Thus, it is possible for a Dutch company to hire a producer in the USA or China, without providing any information to the consumer.

The probability of a non-hormonal supplement containing prohormones is much higher if it is from a company which also sells prohormones. But prohormones were also found in products from companies not selling prohormones (9.6%, Fig. **7**). These companies probably use a prohormone manufacturer for the production of their non-hormonal supplements.

The excretion studies with nutritional supplements containing low amounts of norandrostendione showed positive results for urinary norandrosterone (> 2 ng/ml) for several hours (Fig. **8**). Norandrosterone is the most common metabolite of nandrolone and of all nandrolone prohormones (Fig. **9**). Based on the results of the excretion studies it can be estimated that prohormones of nandrolone in concentrations higher than 1 µg may lead to positive doping results for norandrosterone. Many of the positive nutritional supplements show nandrolone prohormone concentrations lower than 1 µg/g. Nevertheless, the consumption of these nutritional supplements may lead to positive doping results, because the recommended dosage can be as high as 50 g per day. The administration of nandrolone prohormones in amounts higher than 100 µg may result in positive norandrosterone levels for more than 24 hours. It is difficult to predict the maximum urine concentrations of norandrosterone and the time interval above the threshold value of the IOC, because of inter-individual differences in metabolism, and the lack of knowledge about the effect of a long-term application of nandrolone prohormones. The maximum norandrosterone concentration after application of a prohormone-containing non-hormonal nutritional supplement was about 600 ng/ml [5].

The application of testosterone and its prohormones can be detected in doping tests by an increase of the testosterone/epitestosterone ratio. Ratios above 6 may lead to a positive doping result. According to previous experiences, concentrations of testosterone and its precursors, as detected in the present investigation, (also taking into account the recommended dosage and the long-term application) only have a weak influence on the testosterone/epitestosterone ratio or none at all. Nevertheless, an increase of this ratio cannot be excluded, especially in females, who have low concentrations of testosterone and epitestosterone.

Conclusion

The sports community should be aware of the danger of nutritional supplements containing prohibited anabolic androgenic steroids not declared on the supplement label. The present study shows that this is an international problem. The consumption of such nutritional supplements can lead to positive results in doping tests, especially for the nandrolone metabolite norandrosterone. To minimize the risk of contaminations athletes should only buy nutritional supplements from companies which perform a quality check for prohormones, and/or which can guarantee that their products have no contact with prohormones in the production and transportation processes.

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