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Nandrolone metabolites in football players : Utility for *in* and *out* of competition tests

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Abstract

The two major nandrolone metabolites, norandrosterone and noretiocholanolone, are targeted in urine in order to detect doping with nandrolone or precursors.

The actual International Olympic Committee (IOC) cut-off level is fixed at 2 ng/ml for men and 5 ng/ml for women.

Some famous positive cases in France in 1997, in football and judo between others, with low levels of metabolites initiated a debate about the capability for the human body to produce by itself traces of those nandrolone metabolites without any intake of forbidden compounds.

Before and during the 1998 world cup in France, the International Football Federation (FIFA) was strongly concerned by this problematic.

In this respect, a statistical study was held with all players from the first and second divisions of the Swiss football national league. Analyses of the samples were performed with both

MS/MS ion trap and high resolution technologies. With a cut-off level defined at 0.2 ng/ml (twice our detection limit fixed at 0.1 ng/ml), 6% of the players showed traces of nandrolone metabolites in their urine, when collected after a game, whereas before exercise all subjects were below the cut-off limit.

The origin of nandrolone metabolites in the urine after physical effort is still unknown and a conclusion cannot be drawn from the presented study.

There are still two hypotheses:

1. An endogenous production of nandrolone metabolites similar to the production being known during pregnancy.
2. An exogenous intake of nandrolone or precursors with their possible release from fatty tissues only during strenuous physical exercise.
3. An exogenous intake of nandrolone or precursors contained in nutritive supplements ingested before/during the physical effort.

1. Introduction

Nandrolone (19-nortestosterone) is a synthetic anabolic steroid which is widely used in some sports where muscle mass is a deciding factor. This substance is widely spread in body-building, for example. Nandrolone esters (decanoate or phenylpropionate) are generally the most used forms of nandrolone. Intramuscular injection of these nandrolone delayed forms are the usual way for doping. Urinary metabolites excretion is very slow. According to the scientific literature, after a 25 mg nandrolone ester injection, metabolites are detectable for at least 7 weeks (see Le Bizec et al., 1999).

According to our experience, body-builder's urines after a cure of nandrolone injections of several weeks showed the presence of at least one metabolite of nandrolone until 12 months after the last injection .At the end of the excretion, when the concentration was not far from

the detection limits (between 5 and 10 ng/ml), the concentration could vary every day randomly.

Nowadays, oral precursors of nandrolone are available and the clearance from the body is much faster.

The two major nandrolone metabolites, 19-norandrosterone (NA) and 19-noretiocholanolone (NE), are targeted in urine in order to detect doping during the analyses.

The actual IOC cut-off level is fixed at 2 ng/ml of norandrosterone in male's urine and 5 ng/ml in female's ones. This means that the presence of 2 (or more) ng/ml of NA will give a doping positive result. This cut-off value is the result of a consensus decision of several authorities including the IOC sub-commission for doping and biochemistry in sport. It has been recently confirmed at the IOC laboratories meeting in Monaco (IOC Monaco press release dated October 6th, 1999) and by the FIFA itself.

Endogenous production ?

Recent studies have shown that urine of stallions and mare follicular fluid contain endogenous traces of nandrolone, as well as in urine of cows and pregnant boars (see Debruyckere et al, 1990).

Similarly, nandrolone has been identified in human ovarian follicular fluid as a possible intermediate in an enzymatic conversion of androgens to oestrogens (see Dehennin et al., 1987).

Moreover, a pregnant woman might be able to produce nandrolone or norandrosterone, because it was detected in urine of women in their 6th, respectively 14th week of pregnancy (see Van Eenoo et al., 1999 and Mareck-Engelke et al., 1999). The intake of some contraceptive pills might also show the presence of nandrolone metabolites in urine.

Regarding men, no clear answer to the question of the hypothetisized endogenous production of nandrolone metabolites has been given. The world-wide analyses, performed by all the IOC accredited laboratories, of the last few years seem to confirm that the resulting concentration in urine of this possible endogenous production should lay below the cut-off of 2 ng/ml.

Precursors or food contamination ?

The use of injected forms of nandrolone are well known in strength sports, but new oral precursors (19-norandrostendione and 19-norandrostenediol for example) appear now on the grey market of food supplements. The different way of administration has certainly an influence on the metabolism and the time of elimination of the product, but the same main metabolites (NA and NE) are found in the urine. The effects on the performance of such treatments are not well known, but seem at least to open new markets in the athlete population.

It is also known that because of the relatively poor quality control of the production of some food supplements, contamination by these precursors in some cases could not be excluded.

The aim of this study is then to obtain a statistical view of a top level football player's population regarding the occurrence in their urine of nandrolone metabolites. This will be discussed in the perspective of the introduction of out of competition tests for a better understanding of such low concentration cases.

2. Experimental

The technique used for the preparation of samples is similar to that described previously (see Robinson et al., 1999)

Steroids and reagents

The Institut für Biochemie, Deutsche Sportochschule, Köln, Germany, synthesised and kindly gave us the major metabolites of 19-nortestosterone (99% purity), 19-norandrosterone (NA) and 19-norethiocholanolone (NE). The other solvents and reagents were of analytical grade.

Solid phase extraction and hydrolysis

The urine volume necessary to confirm the presence of NA and/or NE in the samples was 5 ml. Once the internal standard (methyltestosterone, 50 ng) was added to the urine samples, a solid phase extraction coupled to an enzymatic hydrolysis was performed. A prewash of the solid phase extraction columns (Bakerbond spe™ C18, 500 mg, J.T. Baker, Phillipsburg, NJ, USA) was done with 5 ml methanol and 5 ml water. The samples were applied onto the columns, and were rinsed with 5 ml of water. The steroids were eluted with four times 2 ml of methanol. The alcohol was evaporated to dryness under an air stream. After that, 1 ml of phosphate buffer (0.2 M, pH 7.0) and 30 µl of E. coli-glucuronidase (5000 units glucur.) were added. The hydrolysis took 1 hour at 50°C or overnight at 37°C.

Basic extraction

Once the hydrolysis was completed the basic extraction included the following steps : 100 mg of solid buffer (Na_2CO_3:$NaHCO_3$, 1:10, w:w) was added to the hydrolisate in order to have a pH contained between 8.5 and 9. Then 6 ml of n-pentane were added and the mixture was shaken for 20 minutes. A centrifugation at 3000 rev/min for 10 min. was done in order to separate both phases. The organic phase was put into a new tube and was evaporated to dryness under a gentle stream of nitrogen.

Derivatization

TMS enol-ether derivatives were formed for GC/MS and GC-Q analysis. The dried samples were dissolved in 50 µl MSTFA *[N-methyl-N-(trimethylsilyl)-trifluoroacetamide]* / DTE *[Dithioerythritol]* / TMIS *[trimethyliodosilane]* (1000:5:5, v:w:v) and heated at 60°C for 30 minutes.

MS-MS analysis

Instrument: GCQ, ion trap Finnigan, electron impact ionisation with 70 eV; column: Ultra-1, fused silica capillary column, WCOT crosslinked methyl silicone, length: 12 m, ID: 0.2 mm, film thickness: 0.33 µm; carrier gas: helium 1 ml/min; injection mode: split; injector temperature: 280°C; interface temperature: 310°C, detector: 310°C, temperature program: initial 180°C for 0.3 min.; program rate: 10°C/min until 300°C, final time 2 min.

SRM mode, isolation of parent ion for 19-norandrosterone and 19-norethiocholanolone 405, fragmentation energy 1.2 eV and mass production between 100 and 410 amu 1 scan/min, isolation of parent ion for methyltestosterone 301, fragmentation energy 1.2 eV and mass production between 100 and 310 amu, 1 scan/min.

Validation of the method was performed as usual in accordance to the good laboratory practice (GLP) in place in IOC accredited laboratories.

Samples

- ***Group I : national league players after game(n=358)***

  With the support of the International federation (FIFA), the swiss national football association and particularly its medical commission co-ordinated the information to the clubs of the two first national divisions. The urine collections were performed under the authority of the swiss anti-doping commission (AOS) and organised in the same way as

regular anti-doping controls.

All the 358 urines were collected over two rounds of the championship.

- ***Group IIa (control group a) : amateur players before and after game (n=137)***

  Several matches in lower graded leagues and in friendly tournaments were selected to provide, on a volunteer basis, urine samples. The collection was in that case organised and supervised by the staff of the laboratory. The range in age was set between 18 and 40 years old. In order to simulate out and in competition tests, urines were provided respectively before and after the game (at least 60 min of active participation).

- ***Group IIb (control group b) : students (n=126)***

  After reading the first results (see section results), it was decided to increase the group without exercise by collecting urines from male students in the same range of age than group IIa.

3. Results

NA and NE concentrations, measured with the MS-MS technique for each group, are described in the following table. Group IIa, represented by 137 amateur players, provided urine before and after a game (min. 60 minutes of effort). The 8 individuals from this group with traces of NA after the game had concentration below 0.2 ng/ml cut-off almost 2 hours before.

a. After competition

NA conc. (in ng/ml)	Group I National league	Group IIa Amateurs
< 0.2	336	129
0.2 - 0.5	9	7
0.5 - 1.0	5	1
1.0 - 2.0	5	0
2.0 - 3.0	3	0
> 3.0	0	0
Total :	358	137

b. Before or without competition

NA conc. (in ng/ml)	Group IIa Amateurs	Group IIb Students
< 0.2	137	126
> 0.2	0	0
Total :	137	126

Table 1. Results of quantifications of NA in several groups after or before competition

It appears from these results that exercise could have an influence on the excretion of nandrolone metabolites, because none of the subjects were excreting any metabolites before effort (Group IIa, football amateur or Group IIb, students), whereas after a game, about 6% (22 subjects on a total of 358 for the Group I and 8 subjects on 137 for Group IIa) did show some traces of NA (>0.2 ng/ml) in their urine.

If in the amateur ranks, most of the subjects with traces of metabolites were in the lower concentrations (<0.5 ng/ml), some of the national league players were higher than 1 ng/ml, three of them being even over 2 ng/ml which represent the limit defined by the IOC to declare a case positive (see detailed results in annex.1)

In its letter of August 7th, 1998, the IOC medical commission gives to the accredited laboratories the analytical criteria for reporting low concentrations of anabolic steroids. Concerning 19-norandrosterone in male urines, the specific gravity should be taken into account. In fact, if the urine specific gravity is higher than 1.020, the relative signal of the

control urine at 2 ng/ml (negative urine spiked with 2 ng/ml NA) should be corrected in order to make the right comparison.

The factor of correction has been defined as the following: when the specific gravity is higher than 1.020, the relative signal from the positive control urine (2 ng/ml NA) should be multiplied by (sg-1)/0.02.

If the concentrations were based only on the non-corrected signals, 3 samples were over the limit. (see Table 2). But when corrected , because 2 of these urines exceeded 1.020 (1.026 and 1.030), they become negative, i.e. under the limit.

This point is interesting at several levels.

- First of all, when using this correction factor, only 1 case of the elite players is over the limit.
- Secondly, this gives a relative weight to the specific gravity measurements when NA values are close to the cut-off.
- Finally, another well known normalisation factor used in clinical chemistry for any urinary concentration is creatinine. When reported to creatinine, it appears that the concentration of subject 22 is higher than for subject 21.

It means that, when using one of the other types of correction and close to the limit, one could be positive or negative.

	Subject 20	Subject 21	Subject 22
specific gravity	1.026	1.016	1.030
creat (g/l)	4.09	3.49	3.85
NA /ng/ml)	2.2	2.2	2.5
NA corr s.g. (ng/ml)	1.7 *	2.7 **	1.7 *
NA corr creat (ng/mg creat)	0.54	0.63	0.65

* *would be declared negative*

** *would be declared positive if this correction (sg ≤ 1.020) was allowed*

Table2. NA conc. from the 3 subjects over the cut-off limit in several mode of calculations

Ratio NE/NA

From our experience, when nandrolone is applied to volunteers in its injected form, the ratio NE/NA is lower than 1 (laboratory data not published). Several hypotheses have been done on the possibility for the male body to produce NA, but not NE and now, this ratio could be a possible way to discriminate endogenous products excretion from exogenous application release.

The individual variability in metabolism and sometimes the analytical difficulty to characterise 19-Noretiocholanolone make any interpretation quite difficult. NA concentration higher than 0.2 ng/ml from the Group I (elite players) have been classified from 1 to 22 and for each of them, the respective concentrations have been drawn (Figure 1.). Even if the origin of these products in the present experiment is not known, the large variability observed in that figure is not helping much for a better understanding. The ratio NE/NA goes from 0.0 to 4.0.

Effect of exercise

The data presented here show quite clearly that exercise has an effect on the urinary excretion of nandrolone metabolites. They do not demonstrate whether they come from any endogenous production or exogenous application. In both cases, it seems at least that their excretion in urine is stimulated by exercise or stress.

Unfortunately, for juridical and practical reasons it was impossible to collect urine before exercise with the elite players (Group I)

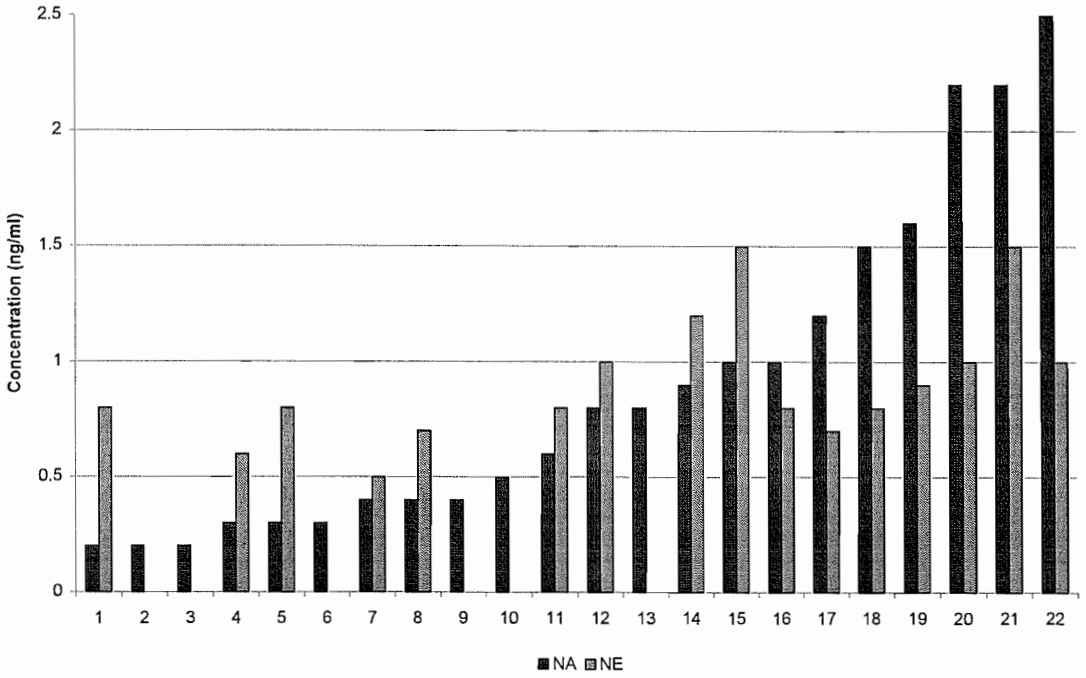

Figure 1. NA (black Blocks) and NE(open blocks) conc. from the 22 elite players over ng/ml NA

4. Discussion

1. Without physical activity no metabolites of nandrolone were detectable in the considered population (amateur + students).

2. Football players of different level of skills present in their urine after physical exercise (physical effort related to game) nandrolone metabolites of higher level than 0.2 ng/ml in 6% of the individuals, whatever the examined group. A level over 2 ng/ml (but not over 2.5) has been detected in less than 1 % of the players.

3. A selected population of amateur football players being examined before and after football games showed no metabolites of nandrolone before the game, however, after a game, 6 % of them had concentration below or equal to 0.6 ng/ml.

4. Based on these results, no conclusion can be drawn on the ratio NA/NE for any discrimination between a potential endogenous production and exogenous application.

5. The origin of nandrolone metabolites in the urine after physical effort is still unknown and a conclusion cannot be drawn from the presented study. There are two hypothesis:
   a. endogenous production of nandrolone metabolites similar to the production being known during pregnancy
   b. exogenous intake with possible storage of the nandrolone or its metabolites in fatty tissue being released only during strenuous physical exercise
   c. potential intake of nutritional supplements containing nandrolone or precursors

6. As nandrolone and nandrolone related substances are available as over-the-counter-drugs without medical prescription further studies are strongly recommended to investigate the possible origin of the nandrolone metabolites in the urine.

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Annex 1. **Individual values from elite players (1-22 out of 336) and amateurs (I-IX out of 137) showing concentrations of nandrolone metabolites above the limit of detection (0.2 ng/ml)**

Individuals	sg	Creat. (mg/ml)	NA	NE	NA/Corr. sg	NE/Corr. sg	NA/creat.	NE/creat
1	1.035	6.62	0.2	0.8	0.114	0.457	0.030	0.121
2	1.029	3.26	0.2	0.0	0.138	-	0.061	-
3	1.028	3.36	0.2	0.0	0.143	-	0.060	-
4	1.035	3.75	0.3	0.6	0.171	0.343	0.080	0.160
5	1.025	4.78	0.3	0.8	0.240	0.640	0.063	0.167
6	1.024	3.60	0.3	0.0	0.250	-	0.083	-
7	1.023	2.57	0.4	0.5	0.348	0.435	0.156	0.195
8	1.022	1.90	0.4	0.7	0.364	0.636	0.211	0.368
9	1.017	2.43	0.4	0.0	0.471	-	0.165	-
10	1.023	3.80	0.5	0.0	0.435	-	0.132	-
11	1.015	2.27	0.6	0.8	0.800	1.067	0.264	0.352
12	1.029	4.74	0.8	1.0	0.552	0.690	0.169	0.211
13	1.026	3.72	0.8	0.0	0.615	-	0.215	-
14	1.024	2.37	0.9	1.2	0.750	1.000	0.380	0.506
15	1.020	3.44	1.0	1.5	1.000	1.500	0.291	0.436
16	1.019	2.36	1.0	0.8	1.053	0.842	0.424	0.339
17	1.030	1.98	1.2	0.7	0.800	0.467	0.606	0.354
18	1.028	4.77	1.5	0.8	1.071	0.571	0.314	0.168
19	1.025	3.99	1.6	0.9	1.280	0.720	0.401	0.226
20	1.026	4.09	2.2	1.0	1.692	0.769	0.538	0.244
21	1.016	3.49	2.2	1.5	2.750	1.875	0.630	0.430
22	1.030	3.85	2.5	1.0	1.667	0.667	0.649	0.260

Individuals	sg	Creat. (mg/ml)	NA	NE	NA/Corr. sg	NE/Corr. sg	NA/Creat.	NE/Creat.
I	1.029	4.95	0.20	0.20	0.138	0.138	0.04	0.05
II	1.026	4.28	0.30	0.00	0.231	-	0.07	-
III	1.020	2.53	0.30	0.20	0.300	0.200	0.13	0.10
IV	1.028	3.86	0.30	0.30	0.214	0.214	0.09	0.08
V	1.028	4.81	0.40	0.00	0.286	-	0.07	-
VI	1.028	3.20	0.40	0.30	0.286	0.214	0.11	0.08
VII	1.031	3.74	0.40	0.60	0.258	0.387	0.12	0.17
VIII	1.029	5.08	0.50	0.20	0.345	0.138	0.10	0.04
IX	1.024	2.85	0.60	0.80	0.500	0.667	0.23	0.28

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KÖLN, DEN 26. September 2000

Lieber Herr Ludwig,

finden Sie als Anlage die erwähnte Publikation sowie eine weitere Publikation, die im Druck ist, zu Kontaminationen von Nahrungsergänzungsstoffen mit Dopingsubstanzen, insbesondere von Steroidhormonen.

Mit Gruß

C. Schänzer

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KÖLN, DEN 26.9.2000

Lieber Herr Franke,

als Anlage übersende ich Ihnen die Publikation zu Nandrolon und Fußballspielern.
Die Arbeti von Kintz habe ich zur Zeit nicht.

Mit freundlichem Gruß

Wilhelm Schänzer