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Origin of elevated levels of norandrosterone in human urine: Half-truths vs. facts

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Introduction

Much attention has been given in the past years to what has been described in the media as a “rash” of nandrolone positive findings reported by the laboratories. As if a newly developed method had just been implemented for the testing of these agents, the findings are heavily challenged by arguing that the metabolites were either endogenously formed or had resulted from the benign intake of food or food supplements. While most of the studies were supportive of the thresholds above which norandrosterone findings are reported, a few indomitable opponents are still firmly committed to proving that the athletes are victims of unfair accusations. In this paper we have summarised the current knowledge related to the excretion of norandrosterone and norsteroids metabolites in human urine. We have objectively studied the results presented in support of the contention that the excretion of norsteroids metabolites had been increased as a result of strenuous exercise and found these to be ambiguous if not of poor quality.

Having studied the excretion of norsteroids from different sources with regards to the relative excretion of norandrosterone to noretiocholanolone and in both the sulfo and glucuroconjugated forms, we were able to conclude that neither could be used to discriminate between the endogenous and exogenous formation. Only the determination of the ¹³C content of the metabolites when possible, can point to the synthetic nature of the substance which has been administered including the ingestion of meat from animal species in which norsteroids are endogenous.

Experimental:

- a) For the purposes of GC-MS analysis, the isolation of the glucuro and sulfoconjugates of urinary steroids was carried out as described previously [1]. The solvolysis of the residue of the enzymatic hydrolysis (β -glucuronidase, *E. coli* type IX-A lyophilised

powder) which has been extracted twice with hexane at pH 11, was carried out with sulphuric acid (4M) in tetrahydrofuran at 50°C for 1 hour and followed by an extraction at pH 9 with diethylether.

- b) For the purposes of the GC/C/IRMS analysis, the combined free and glucuroconjugated steroids were analysed as extracted from the routine procedure when norandrosterone was present in an amount greater than 20 ng/mL while a HPLC purification of the norsteroids had to be carried out on the residue when lower concentrations were found. c) Urine samples collected during routine doping controls from 1993 to 2000 which were found to contain norandrosterone in amounts ranging from 5 to several thousands of ng/mL were randomly selected from the specimens kept frozen d) volunteers who gave informed consent were given a commercial capsule of norandrosterone or meals prepared to contain 300 g of offal from castrated or un-castrated pork. Experiments and results will be fully described elsewhere e) Urine samples were collected during the pregnancy of two consenting volunteers.

Review of the literature:

Norsteroids: administration and excretion

The administration of nortestosterone pharmaceutical preparations and of the other nosteroids which became available for oral self-administration in the past years has been shown to lead mainly to the excretion of norandrosterone, noretiocholanolone and norepiandrosterone, the later being found exclusively as its sulfoconjugate while the first two are predominantly excreted as their glucuronide derivative. While the administration of the long-lasting injectable preparations may be detected for months, the metabolites formed from *per os* administration will be excreted massively in the first hours post administration and therefore will remain detectable for only a few days [2 and references cited herein].

The level of norandrosterone normally found in human urine samples¹

The low excretion of endogenous norandrosterone is normally not detected in human urine samples during routine doping control testing, with the exception of specimens excreted during pregnancy in which levels can go up to around 15 ng/mL [3]. A more sensitive instrumentation, a larger volume of urine and an extensive sample clean-up were needed to detect, identify and quantify endogenous 19-norandrosterone, which in some male specimens

¹ Including athletes' samples

was found at levels varying around 0.01 to 0.32 and 0.05 to 0.6 ng/mL, well below the limit for reporting positive results [2, 4]. In samples collected from females, the level of endogenous 19-norandrosterone is also well below the threshold, lower than 1 ng/mL [5], reaching a mean maximum value of 0.6 ng/mL during the ovulation [6]. The endogenous excretion of norsteroids in males and females seems to be related to the pathway of aromatization. It has been shown to be stimulated by hCG but not related to insulinic stress [7].

Norandrosterone findings reported by the laboratories

The rash of positive nandrolone findings or the extreme number of positive findings is not apparent in our statistics. The actual number of nandrolone findings reported by the I.O.C. accredited laboratories from 1988 to 2000 is summarized in the following table [8] and we observe that the proportion of samples reported positive for the presence of nandrolone (norsteroids) metabolite has been relatively constant from 1993 to 2001. With regards to female athletes' samples, we have tested in Montréal 9586 urine samples from January 1994 to December 2001. Out of these, 13 (0.1%) were reported positive (level greater than 5 ng/mL) for the presence of norandrosterone in an amount ranging from around 7 to 75 ng/mL. The sports involved were weight-lifting, swimming, athletics although principally body building. Tetrahydronorethisterone was present in 468 specimens while low levels of norandrosterone were detected in 367 of them.

Table I: Nandrolone positive findings reported by IOC accredited laboratories.

Year	Total number of tests	Nandrolone positive amples	%
1988	47069	304	0.65%
1989	52371	224	0.43%
1990	71341	192	0.27%
1991	84088	165	0.20%
1992	87808	152	0.17%
1993	89166	227	0.25%
1994	93680	207	0.22%
1995	93938	212	0.23%
1996	96454	232	0.24%
1997	106561	262	0.25%
1998	105250	259	0.25%
1999	118259	293	0.25%
2000	117314	325	0.28%
2001	125701	304	0.24%

Ingestion of un-castrated pork offal.

Although highly improbable, the intake of a substantial amount of un-castrated pork offal in which the presence of norsteroids such as nortestosterone has been measured will result in the excretion of norandrosterone in an amount above the thresholds in the following hours [9]. We will describe in the following section, the preliminary results obtained for the measurement of the ^{13}C content of the norsteroids excreted.

The effect of strenuous exercise.

The relation between strenuous exercise and excretion of norandrosterone has been nothing more than a hypothesis until recently when the results of the only truly controlled study involving athletes clearly demonstrated that it is not the case² [10] and that exercise does not induce nandrolone secretion. Furthermore, the study also confirmed that the baseline levels of norandrosterone in male athletes are ranging from undetectable to a maximum of 0.25 ng/mL (mean value 0.048 ng/mL).

In a paper published in 2001, Robinson et al.[11] having observed the presence of low amounts of norandrosterone in the urine samples of professional football players after a match (an un-controlled study) prudently suggested that the source of the excreted metabolite could be: 1) an endogenous production; 2) the release from the fatty tissues of a previous intake of nandrolone; 3) an intake just prior to the match of nandrolone containing product.

In a first article published in 1999 in the Journal of Chromatography, B. [4], Le Bizec et al. announced in the abstract that “experiments led on athletes showed that after a prolonged intense effort, the 19-NA concentration can be increased by a factor varying between 2 and 4”. The only data available was in fact a single ion chromatogram in which the “peak of norandrosterone”, absolute height, appeared to be higher post exercise without reference to an internal standard, which is certainly not an acceptable practice. Le Bizec et al. [12], from an un-controlled experiment performed on football players, reported that the norandrosterone levels measured in the 385 urine samples of the 40 players all fell below the IOC threshold and that the concentrations measured after the match were “significantly higher than those before games”. Only 4 samples contained norandrosterone in an amount superior to 1 ng/mL,

² This article has been published just after the Workshop of March 2002

the mean value being calculated at 0.097 ng/mL. The authors claimed to have controlled the experiment by carrying blood testosterone and LH analysis, which has nothing to do with the administration of norsteroids.

In an article published again in the Journal of Chromatography, B. in 2001, Galan Martin et al. [13] concluded from their experimentations that 13% of sportsmen (including relatives) and 40% of postmenopausal women were normally excreting metabolites norandrosterone and noretiocholanolone in amounts ranging from 4 to 22 ng per mL. A quick look at the results only demonstrated the poor quality of the chromatograms and the absence of any type of correct identification or respect of minimal criteria. A work of such low quality should never have been published and we are certainly entitled to raise questions and concerns to the editors.

Discrimination between endogenous and exogenous origin

Le Bizec et al. in 2002, this time in Steroids [14] claimed to have found “a promising complementary criterion to more definitively conclude about an athlete’s culpability, especially when nandrolone metabolites are found in the low range.” In this paper, the authors reported that norandrosterone was excreted solely as the glucuroconjugate when nandrolone has been administered while in his same population of 385 football players’ urine samples, up to 30% of norandrosterone which he described as *endogenously* produced, was sulfoconjugated.

A brief review of the protocol used by the authors for the preparation of the specimens points to some analytical bias one being that the hydrolysis of the steroids conjugates was not done properly having been carried out with *Helix pomatia* mixtures in which it is well known that the arylsulfatase present is devoted of activity towards these substrates, specifically 3 α -hydroxy-5 α -sulfates e.g. androsterone sulfate are not hydrolysed. The solvolysis must be used in place of the enzyme preparations as it is known for more than 30 years that these often lack the activity and specificity needed, can bring conversion to other steroids and in complex matrices, their activity may be inhibited by salts for examples [15].

Results and discussion

GC/MS Analysis³

- a) Exogenous origin: our results indicate that the norandrosterone found in randomly selected positive urine samples was mainly present in the glucuroconjugated form, which accounted for 65 to 93% of the total. However, in one specimen collected in 2000, the sulfate was present in a higher amount than the glucuronide e.g. 160 ng/mL and 59 ng/mL respectively. The relative amount of norandrosterone and noretiocholanolone glucuronides was also variable being found in ratios varying from 1:1 to almost 25:1. In the urine samples collected following the administration of a single dose (100 mg) of 4-norandrostenedione to one male volunteer, norandrosterone was excreted in both its glucuro and sulfoconjugated forms. The relative abundance of each drastically changed during the excretion period, the glucuronide being predominant in the first hours, but the slower elimination of the sulfate inverting the proportion in favour of the latter (from 5% to 80%). Norandrosterone was always present in a larger amount than noretiocholanolone (ratios ranging from 2:1 in the first hours to almost 70:1 at the end of the excretion period when noretiocholanolone is almost undetectable).
- b) Endogenous origin: fourteen urine samples collected every two weeks from the 12th to the 40th week of the pregnancy of one volunteer were found to contain norandrosterone in variable amounts ranging from 1.6 to 10.9 ng/mL. Noretiocholanolone was only present in trace amount lower than 1 ng/mL. Again, norandrosterone was predominantly excreted in its glucuroconjugated form which accounted for 80% to 93% of the total.
- c) Ingestion of norsteroids from un-castrated pork offal: no norsteroid was measured in any of the specimens collected either before the administration of meat or following the intake of castrated pork offal. Norandrosterone glucuronide was the main norsteroid excreted in highly variable amounts in the hours following principally the ingestion of kidneys and liver in a lesser extent. Noretiocholanolone glucuronide was also present but in a lesser amount. Sulfoconjugated norsteroids including norepiandrosterone were detected in very low amount and only in the samples originating from the volunteer who excreted those steroids in the larger amount (maximum values were measured at 160 ng/mL of norandrosterone glucuronide in one volunteer).

³ Results will be published elsewhere in detail

GC/C/IRMS Analysis

As described elsewhere [2], the mean $\delta^{13}\text{C}$ ‰ value of endogenous urinary steroids measured in our laboratory is found at around -23.5 (ranging from -19.8 to -26.8) while the ^{13}C content of several norsteroids commercially purchased from 1960s to now, is found in the range of depleted values of -30.3 to -36.0. Not surprisingly, the mean $\delta^{13}\text{C}$ ‰ value of norandrosterone measured in the specimens collected from volunteers' or positive athletes' samples was around -31 while the values excreted during pregnancy, when measurable, were found within the normal range of values e.g. at around -23 [2 and references cited herein]. These results are in agreement with those reported by Mathurin et al. [16]. Although preliminary, the results obtained in the more concentrated specimens collected further to the ingestion of un-castrated pork offal indicate that the ^{13}C content was not distinguishable from the normal values found in humans, being measured at around -23.6.

Conclusion

Other groups have reported previously that further to the administration of norsteroids (Δ^4 and Δ^5 isomers) the excretion of norandrosterone to noretiocholanolone (5α vs. 5β) while most of the time in favour of the 5α -isomer, could be inversed depending upon the substance taken and the time following the administration [17]. Our results indicate that when the norsteroids conjugates are properly measured, norandrosterone glucuronide and sulfate are present in a relative amount which does not enable a distinction between their synthetic or endogenous origin. The use of the isotope ratio mass spectrometry is the only way when the norsteroids are present in a sufficient amount, to prove the origin of the metabolites. That being said, in the past several years, many attempts were made to prove that the thresholds of respectively 2 and 5 ng/mL in male and female specimens were not only consistent with the administration of a norsteroid but could also be found from purely natural sources and not from doping whether deliberate or not. However, only when the specimens were not taken in strictly controlled conditions were levels approaching the threshold of 2 ng/mL measured. With regards to the ingestion of un-castrated pork offal, since norsteroids are normally present in the level of micrograms in the kidneys, liver, heart, not surprisingly, the urine samples collected in the following few hours can contain norandrosterone glucuronide principally in an amount that could be in vast excess of the threshold. Athletes should prudently refrain from eating pork or other animals' offal although the risks of getting an important intake of un-castrated pork kidney are minimal.

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