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REVIEW ARTICLE

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Update on nandrolone and norsteroids: how endogenous or xenobiotic are these substances?

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Abstract Norsteroids are xenobiotics with androgenic and anabolic properties known since as far back as the 1930s. In doping controls, the use of the banned xenobiotic norsteroids is detected in the competitor's urines by the measurement of norandrosterone (19-NA) and noretiocholanolone (19-NE), which are the main metabolites for nandrolone (NT) and most norsteroids with anabolic properties. In 1996, the IOC subcommission "Doping and Biochemistry of Sport" informed the Heads of the "IOC Accredited Laboratories" that the recommended cut-off limit for reporting an offence was to be 1–2 ng ml^{-1} urine for either 19-NA or 19-NE. We will discuss how technical progress in gas chromatography coupled to high-resolution mass spectrometry permitted a dramatic increase in sensitivity with a detection limit of 1 pg ml^{-1} urine, or less, and an assay limit of 20–50 pg ml^{-1} urine, for either 19-NA or 19-NE. As a paradox, norsteroids have been known for decades as not only xenobiotics but also obligatory endogenous intermediates in the biosynthesis of estrogens from androgens in all species, man included. It is this biochemical observation which fed the active scientific and medical controversy initiated in 1998 over the possibly endogenous production of nandrolone and metabolites well over the new IOC's recommended cut-off limit of 2 ng ml^{-1} urine. Notwithstanding the particular technical difficulties attached, we will provide data and discuss the minute endogenous levels detected and measured in man either at rest, after performance or training and compare them to the relatively high levels reported in male athlete's doping controls today. We will also discuss data on the pharmacological effects of some contraceptive therapies containing norsteroids in women. In view of the well-documented noxious effects repeatedly observed after anabolic steroid misuse, the confirmation and implementation of technically proven procedures for reporting norsteroid abuse in sports seems an important enough goal to protect athlete's health against such abuses and justifies up dating the review of the patent scientific and medical experience and knowledge gained over the last 50 years on nandrolone and its minor production in man and woman.

Keywords Doping · Human biosynthesis · Nandrolone · Norsteroids · Sports

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Introduction

Norsteroids are anabolic substances with androgenic properties known since as far back as the 1930s with the work of Butenandt and Tscherning (1934). These substances were designed for treatment of a wide span of haematological and post surgical conditions, cachexia and also in substitutive progestin supplementation. Misuse of nandrolone and related norsteroids, similar to misuse of all anabolic substances, is not a new problem and has been a repeatedly reported feature in sports since the 1960s (Bjorkhem and Ek 1982; Bowers 2002; Catlin et al. 1993, 1997; Donike et al. 1989; Frankle and Leffers 1992; Hartgens et al. 2001; Huhtaniemi 1994; Kuipers et al. 1991; Sullivan et al. 1998; Yesalis et al. 1997) in spite of their inclusion to the list of banned substances in sports since the Montreal Olympic games in Canada 1976. Pharmacokinetics of nandrolone esters injected intramuscularly (i.m.) are very well known (Minto et al. 1997). There are also numerous reports on the side effects of norsteroid (Akhter et al. 1994; Kuipers et al. 1991; Moss-Newport 1993; Rogol et al. 1984) and

tragic misuse (Akhter et al. 1994; Moss-Newport 1993; Sullivan et al. 1998). Nonetheless, strength athletes are indeed convinced that nandrolone increases body weight and lean body mass to a better extent than training alone and helps speedy recovery in between training sessions, although scientific data do not support these claims unequivocally (Kuipers et al. 1991).

The question of the endogenous production of nandrolone has often been raised by athletes themselves, when found positive for nandrolone abuse, in the same way as they claimed endogenous production of epitestosterone (GE) naturally modified the urinary ratio of glucuronides of testosterone over epitestosterone (GT/GE) (Bricout et al. 2003; Catlin et al. 1993, 1997; Franke and Berendonk 1997; Hatton and Catlin 1987; Wilson 1988; Wright et al. 1993). The criteria for the determination of nandrolone or related substances (norsteroids) in urine consists in gas chromatography coupled to mass spectrometric (GC/MS) detection, and measurement of their major urinary metabolites, 19-norandrosterone (19-NA) and/or 19-noretiocholanolone (19-NE) (Donike et al. 1989; Dumasia et al. 1989; Franke and Berendonk 1997; Hampl and Starka 1979; Tabet 1994). We shall discuss these technical improvements since an offence is technically proven when results found are well over the cut off limit of 2 ng ml^{-1} urine, for endogenous release (IOC's implemented regulations) in male subjects and 5 ng ml^{-1} urine in female subjects (Saugy et al. 2000).

Origin of endogenous nandrolone and norsteroids

The first facile synthesis of 19-NA (nandrolone) was reported by Birch (1950) and confirmed by Wilds and Nelson (1953) with estrone as starting material. Since then, the pharmaceutical industry has produced norsteroids for human, and animal, substitutive (hypogonadism), complementary (osteoporosis and haemological diseases) or contraceptive treatment (Frankle and Leffers 1992; Mauvais-Jarvis 1984; Schürmeyer et al. 1984; Segaloff 1963; Wilson 1988).

As a paradox, norsteroids are not only xenobiotics but also obligatory intermediates (Fig. 1) in the biological synthesis of estrogens from obligatory androgens precursors in all species, including man (Desgrez and Malmejac 1970, Dumasia et al. 1989; Farnsworth 1966; Garrett et al. 1991; Hagensen-Jetne et al. 2000; Hillier et al. 1980; Le Bizec et al. 1999; Perel and Killinger 1979; Reznik et al. 1987; Short 1961, 1964; Skinner and Akhtar 1969). However, aromatization of androgens by aromatase is controlled by an enzymic complex ($P450_{arom}$) which presents in most cells, ovaries and placental microsomes excepted, a very small quantitative activity which does not even reach 0.1% biotransformation of the androgen substrates to estrogens (Abraham et al 1969; Bellino and Osawa 1974; Berkowitz et al. 1984; Frisch et al. 1980; Frost et al. 1980; Hillier et al. 1980; Kley et al. 1980; Longcope et al. 1972, 1976, 1983;

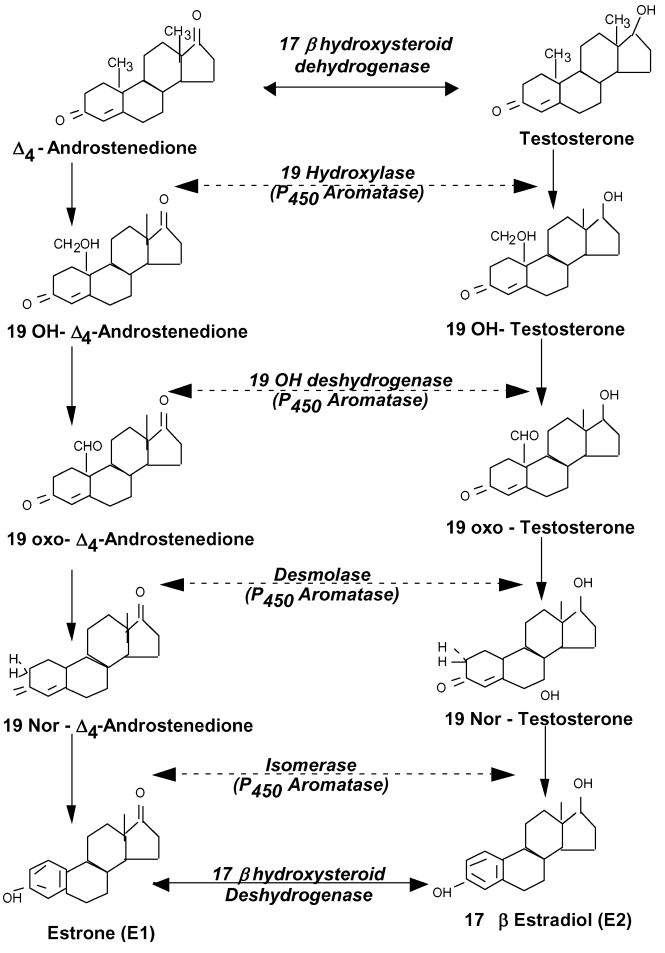

Fig. 1 Estrogen biosynthesis from androgens in human subjects. Norsteroids such as 19-norΔ4 androstenedione and 19-nortestosterone (19-NE) are obligatory intermediates in the biosynthesis of estrogens (From Desgrez and Malméjac 1970 with their kind permission)

McDonald et al. 1967; Nimrod and Ryan 1975; Payne et al. 1976; Perel and Killinger 1979; Ryan 1959; Short 1961; Simpson et al. 1993; Schweikert 1979; Thompson and Siiteri 1974). Although the normal and pathological molecular biology and physiopathological control of the aromatase complex activity are well advanced in animal and human, for both sexes, the actual chemical steps involved in estrogen synthesis are still under discussion. However, production of intermediate norsteroids is universally recognized (Auvray et al. 1998; Desgrez and Malméjac 1970; Dikkeschei et al. 1996; Garrett et al. 1991; Guiochon-Mantel et al. 1999; Harada et al. 1990; Kley et al. 1980; Li and Adams 1981; Macome et al. 1972; Perel et al. 1984; Santen 1990; Simpson et al. 1993; Tilson-Mallett et al. 1983; Weinstein et al. 1974). It is a well-known fact in general enzymology that intermediates normally bound to an enzymic complex may sometime leave these complexes in small amounts and leak out as such (Dumasia et al. 1989; Short 1961). This phenomenon accounts for the presence of some 19 nor-Δ_4-androstenedione (19-NΔ_4) during aromatization of

Δ_4-androstenedione (Δ_4) to estrone (E1) and some 19 nortestosterone (19-NT) during aromatization of testosterone (T) to estradiol (E2), which was first demonstrated in the 1960s (Short 1961, 1964). However, the synthesis of rare amounts of these substances in cells is not proof of their presence in body fluids until it has been properly demonstrated. In fact, only catheterization of either testicular, ovarian or adrenal glands, together with analysis of affluent and effluent blood, could prove beyond any doubt the endogenous origin of such norsteroid production in the general blood circulation and their filtration in urine. Results are yet to be obtained and published.

Research evolution in the detection of nandrolone and norsteroids

In the 1960s and for a certain number of years, due to their poor endogenous yield, the only means to demonstrate the presence of endogenous norsteroids in cells and body fluids was the indirect isotopic labelling of endogenous precursors such as T or Δ_4. Norsteroids were thus reported to be produced in small quantities by testis, prostate, adipose tissue, bone marrow, placenta and ovarian follicles in animals and humans (Dimick et al. 1961; Farnsworth 1966; Frisch et al. 1980; Frost et al. 1980; Osawa and Yarborough 1983; Segaloff 1963, Short 1961, 1964). Segaloff (1963) even proposed a daring hypothesis on the role of 19-NT, stating that due to its very small production rate this could be the active substance in androgen target cells. This substance was thus extensively studied. However, the discovery of dihydrotestosterone (DHT)-selective accumulation and retention in nuclei of androgen target organs (Anderson and Liao 1968, Bruchovsky and Wilson 1968) soon countered this hypothesis. DHT, the synthesis of which is under the control of 5α-reductase, was therefore shown to be the active androgen produced in all androgen target cells. Indeed, in muscle, the presence of the enzyme 5α reductase, contrary to lay knowledge, has been reported (Michel and Baulieu 1976; V. Bricout and F. Wright, unpublished observations). Unfortunately, this finding has very often been overlooked since 3-oxoreductases, which convert DHT to 3α and 3β androstanediols (diols), are very active enzymes in muscle cells and transform all excess DHT to diols in in vitro experiments (Bruchovsky and Wilson 1999; V. Bricout and F. Wright, unpublished observations). However, one has to remember that anabolic and androgenic effects cannot be separated since both are obtained through the unique nuclear androgen receptor (Bergink et al. 1985; Wilson 1988). Recovery in urine of labelled norsteroid metabolites (Dimick et al. 1961) such as 19-NA (5α hydroxylated) or 19-NE (5β hydroxylated) which arise indifferently from either 19-NT or 19-NΔ_4 (and any 19-norandrogen precursor) was also reported in humans (Figs. 2 and 3). Indeed, the ratio for 5α/5β urinary metabolites of norsteroids is similar to that reported for androgens and very close to 1 for endogenous androgens (Mauvais-Jarvis and Bercovici 1968; Mauvais-Jarvis et al. 1969a, 1969b; Donike et al. 1989, 1993), but also for 19-NT (Dimick et al. 1961). Intake of xenobiotic steroids by different routes of administration affects the metabolism of these substances (Mauvais-Jarvis et al. 1969a) and changes the ratio for the recovered 5α/5β urinary metabolites since 5β reductase is an important feature of liver metabolism, whereas 5α reductase is an important feature of androgen metabolism in target organs such as the skin (Mauvais Jarvis et al. 1969b). In the 1980s, specific RIAs were devised to measure either the parent compounds or their metabolites in body fluids (Bjorkhem and Ek 1982; Courtot et al. 1984; Franke and Berendonk 1997; Hampl and Starka 1979). However, with the sensitivity of these techniques (0.5–1 ng ml^{-1} in urine) neither nandrolone nor its metabolites could be detected in body fluids (plasma or urines). At these technical limits, the only exception was reported in pregnant women (Reznick et al. 1987) in whom norsteroid metabolites could be detected after 8–12 weeks amenorrhea, a time when active placental aromatization of androgens to estrogens is known to take over and increase well over 100 times the circulating levels of estrogens (Macome et al. 1972; Thompson and Siiteri 1974). In the 1990s, with the amelioration of the instrumentation devised in the 1980s and the use of more specific and

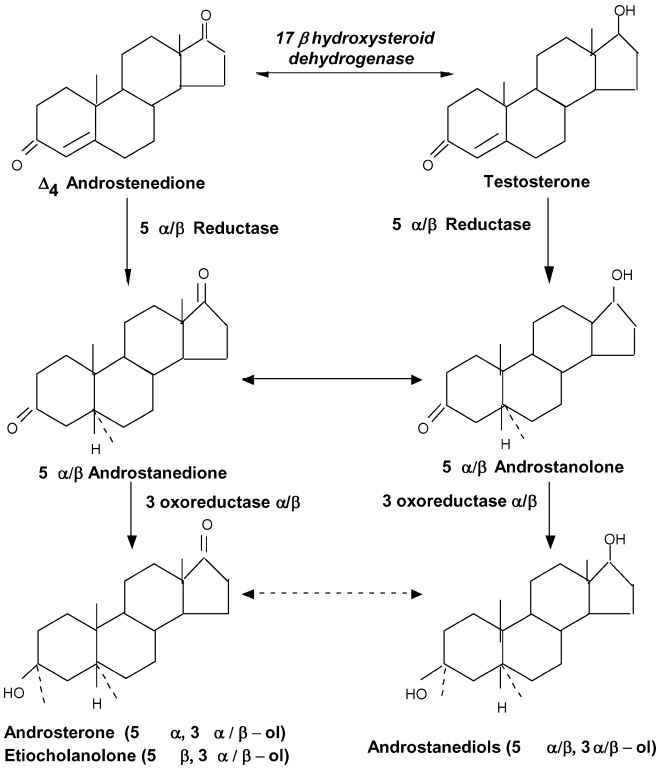

Fig. 2 Natural androgens metabolism in man. Metabolism of testosterone and Δ4 androstenedione follows quantitatively an oxidative pathway which inactivates these androgens. Androsterone (5α,3α) and etiocholanolone, also named isoandrosterone (5β,3α) are the main catabolites recovered in urine. (Mauvais-Jarvis 1984)

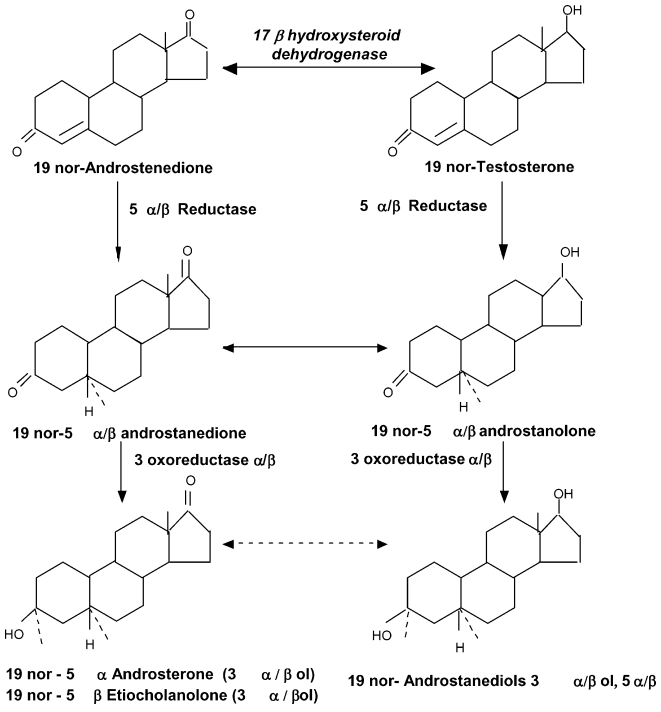

Fig. 3 Nandrolone (19-NT) and norsteroids metabolism in man. Quantitatively, 19-NT and 19 nor-Δ4-androstenedione are also metabolized via an oxidative pathway very similar to that of testosterone (Fig. 2). 19-Norandrosterone (19-NA; 5α,3α) and 19-noretiocholanolone (19-NE, 5β,3α) are the main catabolites recovered in urine (From F Jayle's School of Biochemistry, Faculty of Medicine of the Holy Fathers, Medical School of Paris; Dimick et al. 1961)

sensitive techniques such as GC/MS, the presence of much smaller amounts could theoretically be detected in body fluids (Masse et al. 1989; Tabet 1994). Indeed, with the introduction of gas chromatography coupled to high-resolution mass spectrometry (GC/HRMS) in the selected ion monitoring mode (SIM) or multiple ion trap mass spectrometry (GC/MS/MS/MS), indifferently, a detection limit of 1 pg ml^{-1} urine (at least 1,000 times less substance) was reproducibly achieved (Tabet 1994). These latter techniques are those now in use in National antidoping laboratories accredited by the International Olympic Committee (I.O.C.). By means of these high-resolution techniques, confirmation for up to a maximal value of 0.6 ng ml^{-1} urine for most probably endogenous 19-NA was confirmed in human urine (Dehennin et al. 1999; Le Bizec et al. 1999; Saugy et al. 2000; Schmitt et al. 2002). However, under these basal conditions and with the same techniques, notwithstanding the particular technical difficulties attached to its preparation, 19-NE was more elusive to detect in human urine up to 1998, due to particular interferences depending on the urinary matrix studied, in the area where 19-NE is eluted. Finally, it was reported to be assayed at 20–50 pg ml^{-1} urine (Dehennin et al. 1999; Le Bizec et al. 2002; Schmitt et al. 2002), whereas it was easily detectable, and detected, after controlled administration of nandrolone or norsteroids (Belkien et al. 1985; Hatton and Catlin 1987; Masse et al.

Table 1 Official statistics for doping offences in France as reported by the French Ministry of Youth and Sports. Detection of nandrolone abuse in sports over the last 10 years

Year	Total number of analyses	Total of positives for all substances (Sample A)[a]	(%) (Total)	Total of positives for nandrolone (Sample A)	(%) (Total)
1989[b]	5,300	74	1.40	26	0.49
1990	6,222	68	1.10	22	0.35
1991	7,229	58	0.80	23	0.32
1992	7,999	69	0.86	21	0.26
1993	8,089	142	1.75	16	0.20
1994	7,967	226	2.84	26	0.33
1995[c]	7,081	202	2.85	10	0.14
1996	5,436	156	2.87	30	0.55
1997	5,228	151	2.89	38	0.73
1998	7,113	132	1.86	6	0.08

[a]Results obtained on A samples by the French accredited antidoping laboratory (LNDD) with I.O.C. accredited techniques (GC/MS)
[b]1989–1994: statistical analysis for French plus foreign contractual antidoping controls
[c]1995–1998: statistical analysis limited to French antidoping controls

Table 2 Statistical analysis for IOC's accredited laboratories (results for all 25 laboratories worldwide). Detection of nandrolone offences in sports over the last 10 years. Statistical studies for all analysis performed on sample A; GC/MS technique. With the kind permission of Dr. P. Schamasch, IOC's Medical Director

Year	Total number of analyses	Total of positives for all substances (Sample A)	(%) (Total)	Total of positives for nandrolone (Sample A)	(%) (Total)
1988	47,069	1,153	2.45	304	0.65
1989	52,371	1,206	2.30	224	0.43
1990	71,341	932	1.31	192	0.27
1991	84,088	805	0.96	165	0.20
1992	87,708	993	1.13	152	0.17
1993	89,166	1,222	1.37	227	0.26
1994	93,680	1,278	1.36	204	0.22
1995	93,938	1,516	1.61	212	0.23
1996	96,454	1,569	1.63	232	0.24
1997	106,561	1,779	1.70	262	0.25
1998	113,294	1,926	1.70	259	0.23

1985; Schänzer 1996). Indeed, this is confirmed by the thousands of controls practiced each year by IOC's accredited antidoping laboratories on competing athletes where neither nandrolone metabolite was ever reported as detectable at the limit of 200 pg ml^{-1} urine unless a doping offence was reported and accepted (Tables 1 and 2). Moreover, 10 ng ml^{-1} for a steroid metabolite excreted per millilitre of urine is not an unusual or low amount per se for many natural steroids. Indeed, these are the values routinely measured for medical purposes in patients by the simple RIA techniques for urinary DHT, the active androgen at cellular level, to be followed by testosterone in normal and hirsute women (Bricout et al. 2003; Mauvais-Jarvis et al. 1969b; Wright et al. 1998). The recently published results for nandrolone and norsteroids

metabolites confirm that there is no strange paradox for these nandrolone metabolites, which indeed appear in urine in far smaller quantities than the metabolites of the endogenous androgen testosterone. These nandrolone and norsteroid metabolites are indeed produced by the same route as androgens metabolites from which they are derived. The only difference between the two metabolic pathways (Figs. 2, 3) resides in their metabolic clearance rates (MCRs), since norsteroids are not bound to the specific testosterone and estradiol plasma binding globulin (TeBg or SBP) and are therefore excreted more rapidly (Bergink et al. 1985; Dimick et al. 1961; Mauvais-Jarvis 1984). A recent paper by Reznick et al. (1999) has reported the presence of small quantities of 19-NA in the urine of ten male subjects (calculated rate of production for 19-NA: 3.17 ± 0.35 ng h^{-1}) and of 19-NE in five out of the ten male subjects studied (calculated rate of production for 19-NE: 0.8–4.7 ng h^{-1}). Although this latter study did not confirm that the subjects were free of any norsteroid or steroid tampering, this very small production in man, of possibly endogenous 19-NE and 19-NA, does not challenge the conservatory cut-off limit in use by IOC's accredited antidoping laboratories. In contrast, when one considers the average production of 40–100 ml h^{-1} urine, these results indeed confirm the minute capacities, well under the ng ml^{-1} limit, for a possibly endogenous production of norsteroids in man. Reznick et al. (1999) also reported that this small production was not modified under the influence of the physiological hypoinsulinic stress and only possibly increased after human chorionic gonadotropine (hCG) in parallel to $P450_{arom}$ activity and estradiol production.

In 2000, publication in Köln of studies performed on a large body of football players under the supervision of the Federation of International Football Associations (FIFA) gave very interesting experimental data on possible endogenous production of nandrolone or norsteroids (Saugy et al 2000). The technique used was GC/HRMS at an assaying limit of 0.2 ng ml^{-1} urine for either 19-NA or 19-NE. At this limit, and for 137 amateur football players and 126 students tested at rest, these authors reported the lack of detection of any nandrolone metabolite in any of the subjects studied (Saugy et al. 2000). More recently, Le Bizec et al. (2002) have presented data from 40 professional football players who presented values obtained by even more sophisticated GC/MS method (quadrupole MS coupled to GC) with an assaying limit of 0.02 ng ml^{-1} urine. For this population, mean values for 19-NA and 19-NE were 0.098 and 0.033 ng ml^{-1} urine, respectively, well under the cut-off limit for positive detection during sports doping controls (2 ng ml^{-1} urine).

Nandrolone and norsteroid production after physical exercise

Another question worth addressing in this review is the possible increase in norsteroid production, and of their urinary metabolites, during or after strenuous physical exercise. Indeed, biological dilution of urines after excessive drinking for the purpose of post-exercise rehydration is a normal phenomenon in physiology (whereas tampering with urines in order to dilute them is not), as is concentration of urines after a badly compensated strenuous exercise (whereas tampering with urines in order to concentrate them artificially is not). Physiologists and biochemists are well aware of these facts and have accounted for them when studying steroid metabolites in urinary extracts for medical purposes where creatinine content is always measured and values obtained for metabolites always compared to a reference value per 1 mg creatinine. It is very similar for doping controls in order to treat athletes fairly one way or the other, after strenuous exercise. Devised in order to take into account these high or low urine concentrates of physiological origin, correction factors, for either specific gravity or creatinine content, in the urinary samples studied, have been implemented and in recommended use for the last 20 years at least, as stressed by Donike et al. (1989). This important and little-known technical point having been made, the effects of exercise on possible endogenous production of norsteroids can be reviewed.

A clear-cut answer was expected from the abovementioned inter-laboratory study performed by L. Rivier's team in Lausanne and C. Ayotte's team in Quebec and financed by FIFA's Medical Committee (J. Dvorack in Zurich) on a large population of 621 male subjects (Saugy et al. 2000). As already seen, results obtained for the 137 amateur football players did not show any 19-norsteroid production at rest, at the limit of 200 pg ml^{-1} urine. After exercise, 128 subjects still did not produce any 19-NA or 19-NE, at the same technical limit, whereas 9 of the 137 subjects studied presented a raw increase in 19-NA and 19-NE after competition, together with increased urine concentrations (as measured by either urinary creatinine or specific gravity). Let us analyse these nine potential post-exercise endogenous secretors: one subject produced 0.2 ng ml^{-1} after strenuous exercise, whereas seven showed 19-NA and 19-NE values between 0.2–0.5 ng ml^{-1} and one a value between 0.5 and 1 ng ml^{-1} urine. However, all data but one, when corrected for high specific gravity or creatinine content, showed corrected values largely under 0.5 ng ml^{-1} for both 19-NA and 19-NE and the last subject produced quantities of 19-NA and 19-NE largely under 1 ng ml^{-1} urine. These values for norsteroid metabolites are very small indeed and largely under the cut-off limit of the IOC, nonetheless, none of the subjects studied, not even those showing a small raw increase in norsteroid metabolites after exercise, were controlled in any physiological way in order to ascertain that their hypothalamo-pituitary-gonadal or adrenal axis had not been tampered with prior to the study. Elite football players were also included in this study. Unfortunately, no basal values at rest were obtained for those participating (for juridical reasons). However, let

us review the results obtained after strenuous exercise by these 358 male subjects. Undetectable 19-NA or 19-NE was reported in 336 subjects; 9 subjects had 19-NA and 19-NE values at the limit of 0.2–0.5 ng ml^{-1}; 5 subjects had 19-NA and 19-NE values at the limit of 0.5–1.0 ng ml^{-1}; 5 subjects had 19-NA and 19-NE values at the limit of 1.0–2.0 ng ml^{-1} and 3 subjects had 19-NA and 19-NE values over the cut off limit for positive doping decision with values between 2 and 3 ng ml^{-1} urine. These again are the raw data obtained directly from concentrated urine. After correction for either specific gravity, or creatinine content, all values but one were well under the cut-off limit of 2 ng ml^{-1} urine when specific gravity was used as a correction factor. This single result over 2 ng ml^{-1} urine cannot be regarded as proof of a possible endogenous increase in the production of norsteroids through exercise. First, no proof of an even higher value before the game and strenuous exercise is provided for this ''rare'' subject. Second, as for studies on amateurs, no preliminary or simultaneous study of the hypothalamo-pituitary-testicular/adrenal axis was presented for this or the 22 subjects presenting measurable 19-NA and/or 19-NE as compared to the vast majority of the 336 individuals who were under the technical limit of the assay. Since data presented are not complete, no proof for a patent increase in either endogenous production or effect of exercise on norsteroid production is therefore presented in this paper. Indeed, the authors themselves reviewed the scientific limits of their study and advanced some interesting hypotheses for further analysis. Firstly, they admit that ''an endogenous production of nandrolone metabolites similar to the production known during pregnancy is hard to explain in normal male subjects''. Secondly, they suggest that ''an exogenous intake of nandrolone or precursors, stored in fatty tissues, could possibly be released from these fat cells only during strenuous physical exercise''. This last point presents an interesting alternative hypothesis since strenuous exercise is known to increase fatty acid release and use for energy purposes by muscle cells under aerobic conditions. Thirdly, ''an alternative explanation could be that of an exogenous intake of nandrolone or precursor(s) contained in nutritive supplements ingested either before or during the physical effort''. This hypothesis could also present an interesting alternative to endogenous production since steroids tampering with such nutritive supplements have already been well publicized and confirmed by sportsmen (and women) themselves and studied by antidoping laboratories (Ayotte et al. 2001). Although very interesting, these results need to be confirmed with further complete clinical investigations.

More recently, data have been provided for 29 male athletes studied before and after two types of exercise (Wingate test and treadmill test). The technique used was also GC/MS in SIM mode with a limit of detection for 19-NA and 19-NE established at 5 $pg.ml^{-1}$ urine (Schmitt et al. 2002). Neither type of exercise significantly modified either urinary 19-NA or 19-NE, regardless of whether creatinine was taken into account or not for this population where dehydratation was very controlled and limited. These results support the contention that exercise does not appear to modify significantly the minute production of endogenous norsteroids which is well under the cut-off limit designated by the IOC medical committee for male athletes (2 ng ml^{-1} urine).

Natural androgen production after exercise in men

The same question as discussed above must also be addressed for natural androgens since they are the source of nandrolone and norsteroids. The answer is well illustrated in the literature since all authors agree with Morville's findings that testicular androgens decrease after exercise (Morville et al. 1979; Wright et al. 1998). This proven fact, which led some medics to advocate steroid supplementation for ''tired or exhausted'' athletes in order to facilitate their recovery, is valid for both total androgen production and their urinary metabolites. This physiological fact therefore confirms and gives substance to the present observation that endogenous nandrolone and norsteroids were possibly never seen (Table 3) or reported, with undeniable scientific proof, as physiologically increased over the cut-off limit of 2 ng ml^{-1} urine after international sports competitions (false positive). Indeed, in case of a very concentrated miction, a raw value could appear as increased, but correction for creatinine content or specific gravity denies this crude observation. This is also confirmed by results provided by both IOC (Table 1) and the French antidoping laboratory, LNDD, (Table 2) for the population usually screened in antidoping controls, since these generally take place after competition and strenuous physical exercise.

Studies on sportswomen

Since increased concentrations of 19-NT were found in the plasma of pregnant women after the second trimester of amenorrhea (De Boer et al. 1992; Dehennin et al 1987; Reznik et al. 1987), and since production of endogenous 19-NT involves aromatization of Δ_4 androstenedione to estrone (E1) or testosterone to estradiol (E2), the cut-off limit for female athletes is 5 ng ml^{-1}urine. Very few studies have been made with sportswomen. However, a recent study performed on 360 samples issued by 12 women who collected their urinary mictions in order to evaluate the possible fluctuation of 19-NT concentration throughout the hormonal variations which characterize the menstrual cycle presents interesting data. No modifications were reported between follicular and luteal phases of the cycle for the tested women and values were all under 0.8 ng ml^{-1} (Hagensen-Jetne et al. 2000). Another more recent study (Bowers 2002) has reported that out of 251 sportswomen

Table 3 Urinary androsterone as compared to 19-norandrosterone (GC/MS/HRSIR). With kind permission of Nicoletti (1998, Rome Antidoping Laboratory): 15th Köln Workshop on Dope Analysis. Sport und Buch Strauß Edition Sport, Köln

Athletes' samples	Androsterone screening values (ng ml^{-1} urine)	Norandrosterone screening values (pg ml^{-1} urine)
Male		
1	2,700	130
2	3,000	48
3	3,140	Not determined
4	3,300	130
5	3,339	78
6	3,700	35
7	3,702	42
8	4,080	30
9	4,200	150
10	4,200	90
11	5,200	40
12	5,200	108
13	6,300	116
14	6,600	59
15	8,000	150
16	8,000	110
17	8,200	100
18	8,200	Not determined
19	10,000	155
20	12,000	126
Female		
1	970	100
2	1,110	300
3	1,272	100
4	1,584	Not determined
5	2,028	Not determined
6	2,086	90
7	2,090	70
8	2,245	Not determined
9	2,650	280
10	2,677	Not determined

tested at the Nagano Olympics only 37 (13.7%) had detectable concentrations of 19-NT, of which none exceeded the 5 ng ml^{-1} urine cut-off limit. Of the eight with concentration between 2 and 5 ng ml^{-1} urine, two were pregnant and two were using an oral contraceptive containing norethisterone (which can be specifically differentiated by further analysis of urinary metabolites); the others remain to be explored. These results seem to confirm the personal communication from F. Nicolletti in 1998 (Table 3) and her statement that the actual limit for sportswomen was of a very conservative type (Nicolletti 1998). In conclusion, there is no evidence to suggest that the conservative cut-off limit applied by IOC's Sports Federations for nandrolone detection in women is inappropriate and could lead to false positives.

Pharmacological studies on norsteroids and steroids in general

Pharmacological substances are created in order to permit medical or veterinary treatment of patients or animals presenting a default in the related endogenous substances. The posology in use only permits substitutive compensation. This physiological use has proved its beneficial effects for treatment of a lot of disorders in man and animal (Mauvais-Jarvis 1984; Bhasin et al. 2003). Misuse of these substances occurs when they are used in uncontrolled large doses alone or in combination (stacking), with the sole purpose of improving performance and no medical concern whatsoever (Wilson 1988). Misuse of anabolic substances in sports is detected with coupled GC/MS techniques after appropriate extraction and purification of the extract. Before any such drug is put on the market an extensive study of the various properties of the drug is made by the pharmaceutical firm, including pharmacological studies on its metabolism at later stages.

Metabolism of exogenous nandrolone was first studied by Engel et al. (1958). The first point consistently observed for nandrolone and norsteroid metabolism was that they were catabolized more rapidly than testosterone, but in exactly the same way (Dimick et al. 1961; Minto et al. 1997). This difference in the timing of the excretion of similar norsteroid metabolites is due to the absence of binding of nandrolone and norsteroids to the plasma protein TeBg, which specifically binds testosterone and estradiol and hampers access by the metabolizing enzymes (Hampl and Starka 1979; Mauvais-Jarvis 1984). Likewise testosterone esters and other anabolic substances, nandrolone and norsteroids are thus metabolized as a function of the length of the ester attached to their 17 carbon in the α position (17α) and of the administration route (Mauvais-Jarvis et al. 1969a, 1969b; Minto et al. 1997). Indeed, steroid metabolism always follows certain biological constraints which have to be taken into account when pharmacological studies are made. We will summarize them here: (1) accounting for the volume of the cellular compartment(s) involved from synthesis (or administration) to blood diffusion and also possible concentration in target organs; (2) accounting cellular distribution of enzymes specifically attached to their metabolism; (3) accounting for chemical stability of the substance studied which, for steroids, depends on their 5α or 5β structure, which modifies their spatial configuration (stereospecificity) and on the length of the ester attached; (4) accounting for the mode of transport from blood to target cells/organs and back to blood; (5) accounting for the glomerular filtration and excretion of metabolites in final urine collected in the bladder. To be more specific, metabolism of xenobiotic steroids is modified according to their penetration route in a similar way to that of endogenous steroids (Mauvais-Jarvis et al. 1969a, 1969b). For example, liver, which is the first organ to take up anabolic substances after oral administration, is an organ rich in 5β reductase. It will therefore favour 5β metabolites over 5α metabolites. Muscular injections or percutaneous administration, where anabolic substances reach an androgen target organ rich in 5α reductase will first produce more

5α-reduced metabolites than oral administration. The $5\alpha/5\beta$ ratio for endogenous androgen metabolites has been shown to be close to 1 in man, whether athlete or not (Donike et al. 1989; Mauvais-Jarvis et al. 1969b; Schänzer 1997). The ratio of $5\alpha/5\beta$ metabolites must therefore be considered in pharmacological studies according to the route of administration of the xenobiotic substance.

Could the urinary ratio A+E/19-NA (or A+E/19-NA+ 19-NE) be successfully applied in doping controls?

Androsterone (A) and etiocholanolone (E) are the main metabolites of T and Δ_4 either endogenously produced or of xenobiotic origin. On the other hand, 19-NA and also 19-NE represent the possible result of endogenous $P450_{arom}$ activity, as measured by plasma estradiol levels for example. This activity, which induces gynecomastia when in excess in man, is strikingly low in 46XY humans. However, 19-NA and 19-NE are also metabolites of xenobiotic nandrolone or norsteroids. Tampering with both androgens (A + E) and norsteroids (19-NA + 19-NE) therefore leads to an A + E/19-NA ratio with no meaning whatsoever as both sides of the ratio are the result of tampering with nature and could very well compensate one another to give rise to a meaningless "normal" ratio. Since both androgens and norsteroids seem to be potent, misused substances, this obviously is incorrect and is the reason why this ratio was never acknowledged by IOC's antidoping officials, in contrast to the ratio testosterone/epitestosterone in urine, in which both endogenous substances have been well studied and quantified and endogenous data for both are well known (Bricout et al. 2003; Donike et al. 1989; Wright et al. 1993).

Could contamination in human urine arise from spiked food?

Can consumption of meat from norsteroid-treated animals give rise to positive nandrolone cases? This question was first put forward by researchers from the Ghent antidoping laboratory (Debruyckere and Van Peteghem 1993) and the answers are controversial.

Pharmacokinetic studies of the urinary excretion of nandrolone (N-T) metabolites, following consumption of N-T-fortified food, showed dose-dependent excretion of 19-NA for several hours (Debruyckere and Van Peteghem 1993). Urinary 19-NA was also studied after consumption of raw minced meat purchased randomly at butchers in Belgium (150 g). Detected values ranging from non-detectable (48 subjects) to 3.79 ng ml^{-1} urine (2 subjects) were reported (Debruyckere and Van Peteghem 1993). These striking results are well over the cut-off limit for positive detection for the IOC (2 ng ml^{-1} urine). The authors suggested that these results originated from minced meat contaminated by animal injection sites, whereas residues in first quality tissues, such as steak, were far too low to interfere with urinary doping controls, thus cautioning athletes on their menu. However, one question arises on gustative qualities of nandrolone (or any other anabolic steroid)-contaminated meat comprising injection sites since these substances are known to present a very bitter and foul taste which should be easily detected by consumers. Moreover, results as presented by antidoping tests in athletes do not present this high (2/50, i.e. 4%) statistical range (Le Bizec et al. 2002; Saugy et al. 2000).

For Le Bizec et al. (1999, 2002), although some edible specialties (e.g. wild boar testicles) are indeed rich in nandrolone and could lead to an increase in nandrolone metabolites in urines after consumption, only unusual quantities (100+ kg) would induce modification over the IOC's cut-off limit of 2 ng ml^{-1} urine. They confirm that with increased veterinary controls on all animal carcasses produced at slaughter houses this hypothesis is very unlikely. Moreover, with the spectre of the Creutzfeld Jacob's epidemic and the EEC regulations on steroid abuse in cattle, little now escapes the alert veterinary surgeon in charge of inspection of carcasses, not to mention injection sites which are destroyed immediately.

However, from the sale of energy-rich food supplements spiked with steroids (for improved muscle growth), there arises the possibility of illegal malpractice. Although these supplements do not have the status of endogenous substances, they are certainly not authorized in France by the French Food and Drug Administration, nor in Europe, and should not be used by athletes who would risk being penalized by their sport's governing bodies, even in cases when they were unaware of such tampering, since they are legally responsible for their eating habits. Metabolites originating from such spiked sources would, of course, present the same features as metabolites from an endogenous source with the exception of the $5\alpha/5\beta$ urinary ratio favouring the 5β metabolites obtained in increased amount after ingestion, liver being the first organ accessed by this oral administration route.

Indeed, in a study by Ayotte et al. (2001), discrimination between synthetic and natural metabolites was shown to be possible by using a combination of GC-MS with GC-C-IRMS or possibly analysis of the status of the quantitative urinary metabolites conjugates which present themselves as either glucuro- or sulfo-conjugates (Le Bizec et al. 2002). Moreover, isotopic analysis of the nandrolone or norsteroid metabolites could also be used to distinguish between endogenous and xenobiotic origin, but this would have to be performed on very large volumes of urine, possibly 100 ml or more, since these substances appear in such small quantities as urinary metabolites. However, technical progress in isotopic analysis could make this legally indisputable technique possible as is the case for GT/GEpiT (Aguilera et al. 1996).

Conclusion

Nandrolone and norsteroids have been well known as drugs since the 1940s. They have been extensively studied in both animals and humans for the last five decades (Birch 1950; Engel et al. 1958; Dimick et al. 1961; Schürmeyer et al. 1984; Belkien et al. 1985; Dumasia et al. 1989; Minto et al. 1997; Bhasin et al. 2003). They are also obligatory intermediates in estrogen biosynthesis, in both animals and man (Short 1961; Skinner and Akhtar 1969; Berkowitz et al. 1984; Courtot et al. 1984; Bergink et al. 1985; Dumasia et al. 1989; Garrett et al. 1991). However, minute amounts of nandrolone leakage from cells containing the $P450_{arom}$ aromatase enzyme, which control its release, only leads to a very small endogenous production of their metabolites (19-NT or $19\Delta_4$) in urine. This minute endogenous production of nandrolone and norsteroids is in fact dependent on estrogen production in both male and females (Skinner and Akhtar 1969; Desgrez and Malmejac 1970; Osawa and Yarborough 1983; Garrett et al. 1991; Reznik et al. 1999). This explains the possible detection of nandrolone or metabolites (19-NA and 19-NE) in pregnant women after the second trimester of amenorrhea, a time when placental enzymes take over the estradiol production necessary for the maintenance of pregnancy (Short 1961; Reznik et al. 1987).

The controversy over endogenous or xenobiotic levels of nandrolone metabolites in the urine of athletes found positive over the new limit of 2 ng ml^{-1} urine, as determined in 1996 by the IOC's Medical Commission, is explained by the confusion of athletes and sports-related bodies over the new possibilities of the analytical techniques and procedures in use in antidoping laboratories. Indeed, a gain in sensitivity of 1,000× (from 1 ng ml^{-1} 19-NA to 1 pg ml^{-1} 19-NA) for the new detection techniques benefitting from the new GC/HRMS technique over a period of 5 years (Courtot et al. 1984; Tabet 1994; Franke and Berendonck 1997; Schmitt et al. 2002) may be difficult to comprehend. Published data now show that the maximum level of endogenous production of nandrolone or its metabolites is well under 0.6 ng ml^{-1} in normally concentrated urine or in over-concentrated urine after correction for specific gravity or creatinine content, a correction factor which takes into account low or high urine concentrations (Reznick et al. 1987; Masse et al. 1989; Saugy et al. 2000; Le Bizec et al. 2002; Schmitt et al. 2002). Moreover, dehydration does not increase aromatization of androgens since it is, on the contrary, increased by body fat (Frost et al 1980).

Strenuous exercise, either in strength or endurance athletes, tends to lower androgen production in man (Morville et al. 1979; Bhasin et al. 2003) and does not seem to modify nandrolone production (Schmitt et al. 2002). Indeed, nandrolone concentrations could appear higher in abnormally concentrated urine (Le Bizec et al. 1999). However, correction for the urine concentration itself (specific gravity or creatinine content) produces results close to the average 0.6 ng ml^{-1} urine. This corrective measure is always used in human biology and clinical medicine and is always implemented in doping controls as demonstrated and recommended by Donike et al. (1993). After exercise nandrolone metabolites are thus well under the cut-off limit of 2 ng ml^{-1} urine (Saugy et al. 2000; Schmitt et al. 2002).

Nandrolone assays for women are a different issue, even with the new techniques, since endogenous production is normally close to undetectable levels except after the second trimester of pregnancy (Reznik et al. 1987; De Boer et al. 1992; Schmitt et al. 2002). However, some contraceptive treatments contain norsteroids, which are metabolized into the same urinary end metabolites. This normal phenomenon is taken into account, leading to a higher cut-off limit in female athletes of 5 ng ml^{-1} urine (Nicolletti 1998; Hagensen-Jetne et al. 2000).

Kinetics of metabolism and excretion of xenobiotic nandrolone or norsteroids in man have been well studied (Bjorkhem and Ek 1982; Belkien et al. 1985; Bergink et al. 1985; Minto et al. 1997; Bhasin et al. 2003). The results point out to a faster excretion of these substances as compared to testosterone. Nandrolone, in contrast to testosterone, is not bound to the specific plasma protein TeBg. However, a small accumulation of nandrolone or norsteroids has been observed in fat tissues and could explain some delayed release which cannot be ignored (Frost et al. 1980; Saugy et al. 2000).

Nandrolone and norsteroids are metabolized by the same intracellular enzymes as testosterone (Dimick et al. 1961; Desgrez and Malmejac 1970). This metabolism leads to the production of 5α (19-NA) and 5β (19-NE) metabolites which are normally excreted in urine in similar quantities giving a ratio of approximately 1 (Mauvais Jarvis et al. 1969b; Donike et al. 1989; Schänzer 1997). Since 5α-reductase is an enzyme specific of androgen target cells and 5β-reductase of liver the 5α/5β ratio is modified in urinary metabolites after oral administration of nandrolone, thus favouring 5β metabolites (19-NE) and explaining some of the results of doping controls for athletes in 1998. Indeed, 19-NE was reported in higher quantities than 19-NA (Le Bizec et al. 1999). However, a technical problem arises in GC/MS or GC/HRMS techniques for measuring 19-NE (Le Bizec et al. 1999), since this metabolite is eluted in a chromatographic zone which is very dependant on the quality of the urinary matrix being studied. In normal controls, 19-NE is often assayed in quantities well under those of 19-NA, therefore leading to a very low 5α/5β ratio instead of the expected value of 1 (Schmitt et al. 2002). This is completely reversed in nandrolone or norsteroid oral abuse, probably due to the increase in the quantities being recovered and assayed by this sophisticated method (data from publicized results on positive athletes where values averaged 5–10 ng ml^{-1} urine for 19-NA and 10–15 ng ml^{-1} urine for 19-NE).

Indeed, nandrolone- or norsteroid-spiked food could lead to the excretion of increased 19-NA or 19-NE

metabolites in the urine of concerned subjects. The same applies to such delicacies as wild boar testes. However, the quantities needed to be ingested to give a positive doping control would be enormous (Le Bizec et al. 2002), since a portion of these substances is excreted directly in faeces (Dimick et al. 1961). Moreover, androgens and norsteroids have a bitter taste due to their chemical structure and large quantities of these substances should be detected by the consumer.

Finally, the recommendations made in 1996 by Pr Jorge Segura for IOC's Technical Scientific Commission therefore remain valid, even conservative, with a cut-off limit for the endogenous production of nandrolone (or norsteroids) metabolites of 2 ng ml^{-1} urine in male and 5 ng ml^{-1} urine in female athletes. Positive nandrolone (or norsteroid) doping should be reported for any value above this cut-off limit for either norsteroid metabolite (19-NA and/or 19-NE), whether at rest or after strenuous exercise. Moreover, even if ethic and public health considerations are not the primary concern of this review, we would fail as medical biochemists by not cautioning readers on the numerous medical reports on adverse effects of all abuses of anabolic substances, whether androgens or norsteroids (Frankle and Leffers 1992; Catlin et al. 1993; Yesalis et al. 1997; Sullivan et al. 1998; Wilson 1988; Froehner et al. 1999). These provide sufficient proof of the deleterious effects of their abuse, especially on cardiac (Sullivan et al. 1998) and testicular target organs (Froehner et al. 1999) but also behavioural and psychological issues (Frankle and Leffers 1992; Yesalis et al. 1997). In conclusion, where experience and biological certainty contribute to prove beyond any doubt that this very conservative cut off limit of 2 ng ml^{-1} urine is indeed already a physiologically stretched value (normal mean value in men 0.6 ng ml^{-1} urine), we must press for its implementation in order to protect the health and well-being of all sportsmen.

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