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Evidence for the presence of endogenous 19-norandrosterone in human urine

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Abstract

In 1997, in the scope of antidoping control in sport, a not inconsiderable number of urine analysed by official laboratories revealed the presence of 19-nortestosterone (19-NT: 17β-hydroxyestr-4-en-3-one) metabolites: 19-norandrosterone (19-NA: 3α-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (19-NE: 3α-hydroxy-5β-estran-17-one). These repeated results on a short period of time generated some investigations and especially the verification of the possible production of these metabolites by an unknown endogenous route in adult entire male. Some experiences were led on different persons known to be non-treated with steroids and more precisely with nandrolone. Extractive methods were developed focusing on their selectivity, i.e. searching to eliminate at best matrix interferences from the target analytes. Gas chromatography coupled to mass spectrometry (quadrupole and magnetic instruments) was used to detect, identify and quantify the suspected signals. Two types of derivatization (TMS and TBDMS), a semi-preparative HPLC as well as co-chromatography proved unambiguously the presence, in more than 50% of the analysed urine ($n=40$), of 19-NA at concentrations between 0.05 and 0.60 ng/ml. 19-NE was not detected with the developed methods (LOD$<$0.02 ng/ml). 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. Even if some complementary researches have to be done in order to determine the maximal physiological level of 19-NA and 19-NE, these results should considerably change the strategy of antidoping laboratories.

Keywords: Antidoping control; 19-Norandrosterone; 19-Noretiocholanolone

1. Introduction

The biological activity of 19-norandrostane derivatives was first studied by Dirscherl et al. in 1936 [1,2] after synthesis of this class of compounds as a result of using the benzene ring and carbonyl group hydrogenation of estrone [3]. 19-Nortestosterone (17β-hydroxyestr-4-en-3-one) also named nandrolone was synthesised for the first time by Birch [4] in 1950 and by Wilds and Nelson [5,6] in 1953. More recently, new synthetic procedures have been developed for the preparation of 19-norsteroids using, as starting material, compounds of the androstane serie [7–9]. The substitution of the C-19 methyl group by a hydrogen atom in testosterone changed appreciably the ratio between anabolic and androgenic activity. Certain 17-esters of nandrolone are much more clinically interesting than the compound itself, e.g. the phenylpropionate [10] or the decanoate [3]. This androgenic steroid is used in

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veterinary as well as in human medicine in cases of protein deficiency diseases, osteoporosis or burns. The use of this anabolic steroid as a doping agent to improve muscular strength and performance was rapidly extended in sport and horse racing because of its slight androgenic activity. 19-Nortestosterone has been also employed as a growth promoting agent to accelerate weight gain and improve feeding efficiency in animals [11–13].

Metabolism studies related to nandrolone or its esters have been realised in different species. Its metabolism in man was first investigated in 1958 by Engel et al. [14]. Two main metabolites were isolated after administering nandrolone to a postmenopausal breast cancer patient: 3α-hydroxy-5α-estran-17-one (19-norandrosterone) and 3α-hydroxy-5β-estran-17-one (19-noretiocholanolone) (Fig. 1). Although other metabolites are produced (19-norepiandrosterone), the control of their illegal administration is mainly based on the two first molecules [15–19]. According to Schänzer [19], metabolites of 19-nortestosterone in man are produced in the ratio 5α/5β of 72:28 ($n=1$), instead of 53:47 for testosterone. Houghton et al. studied the metabolism of 19-NT in horse and identified in urine the presence of glucuronic acid conjugate of 5α-estrane-3β,17α-diol, 5α-estrane-3β,17α-diol, 5α-estrane-3β,17β-diol and epinandrolone [20,21]. In bovine, the metabolism studies showed that the most abundant metabolites were epinandrolone, 5α-estrane-3β,17α-diol and 5β-estran-3α-ol-17-one whatever the administration route [23,24]. The most diagnostic analyte used to prove the illegal administration of 19-nortestosterone appears to be epinandrolone.

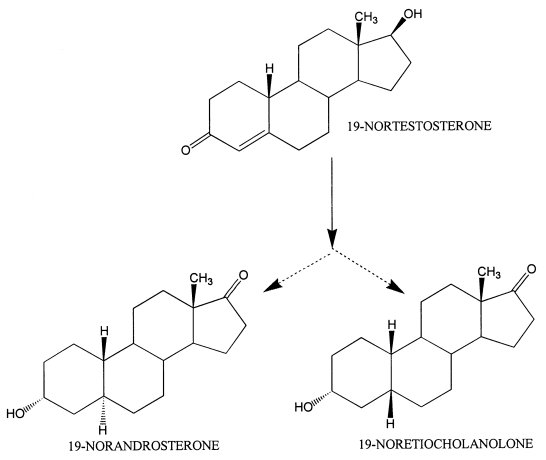

Fig. 1. Structures of 19-nortestosterone, 19-NA and 19-NE.

In the horseracing world, Courtot et al. [25] and Houghton et al. [26] found in 1984 that 19-NT was normally present in the urine of stallions; these preliminary results were confirmed later [27,28]. Presence of nandrolone in equine follicular fluid was also demonstrated during the same period [29,30]. The natural production of 19-NT and 19-norandrostenedione in the follicular fluid [29] and 19-NT in the plasma [31] of pregnant women was observed as well.

Belgian and Dutch researchers found that 17β-nandrolone was endogenously produced by intact boar [32–35]; high concentrations (up to around 1 ppm) can be found in urine and minor levels were measured in edible parts. The first suspicion that 19-NT could also be endogenic in cattle was based on an artefact coming from 17α-testosterone (which was interpreted as 17α-nortestosterone because of poor methodology specificity) in the urine of male veal calves [37]. Meyer et al. explained in 1989 positive results in veal calve urine due to 19-nortestosterone in the feed, resulting from 19-NT contaminated bovine fat used for milk replacer production [38,39]. Endogenous production hypothesis was made in 1991 by Vandenbroeck et al. in pregnant cow thanks to RIA analyses [36]. Meyer et al. identified in 1992 17α-nandrolone in the periparturient cow and the newborn calf urine [40]. Because of the important consequences for veterinary inspection in the EU, the EEC/DGVI asked officially to some official laboratories to confirm these results. The results obtained by GC–MS converged to the same conclusion: 17α-nortestosterone was unambiguously present in the cow *peri-partum* and the neo-natal calf urine [37,41,42]. Clouet et al. demonstrated the same phenomenon in pregnant sheep [43].

From all these discoveries, the new status of endogenous compound for nandrolone or its metabolites generated important consequences for the regulatory control. The strategy was systematically reviewed taking into account ratio between metabolites or concentrations. For instance, in horse, the international threshold for nandrolone in equine urine is reached when the ratio of 5α-estrane-3β,17α-diol to estr-5(10)-ene-3β,17α-diol exceeds one [22]. For

meat inspection purposes, boars are no longer sampled for nortestosterone analysis; pig meat and derivative products are no longer controlled on 19-NT in trade between EU countries [37]. When epinandrolone is found in cow urine, a diagnostic of gestation is generally realised to exclude the possible endogenous origin.

Recently, a not inconsiderable number of nandrolone positive samples in adult males were found in a human anti-doping laboratory. The surprising results were not linked to the frequency of the positive cases but more to the sports which until that time had never been concerned by this scourge. Indeed, the involved disciplines were not until now linked to anabolic steroid consumption: judo, handball, football, and figure skating, etc. After having reproduced, with an independent method, the results of the first laboratory, the possible endogenous production of 19-NT main metabolites, i.e. 19-NA and 19-NE, was explored [44,45]. For this purpose, a sensitive and specific GC–MS method was developed and validated on real samples. Healthy volunteer urine were then analysed at different moments of the day. Furthermore, the follow-up of individual male adults was realised before and after sport events. Finally, the profiles of more tendentious urine always subjected to some interrogations have been shown and discussed.

2. Experimental

2.1. Reagents and chemicals

Most of the reagents and solvents were of analytical quality and purchased by Merck (Darmstadt, Germany) and Solvants Documentation Synthesis (SDS, Peypin, France). *Helix pomatia* juice was from Biosepra (Villeneuve la Garenne, France) and β-glucuronidase (*E. Coli*) was from Sigma (St. Quentin Fallavier, France). The solid-phase extraction (SPE) C18 columns (2 g of phase) were from Varian (Harbor City, CA, USA) and the silica gel G60 was from Merck. The immuno-affinity column raised again diethylstilbestrol (DES), 19-NT and zeranol, was from Genego, Gorizia, Italy. The derivatization reagents *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), *N*-methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), *tert*-butyldimethylchlorosilane (TBDMCS) and trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTE) was from Aldrich (Milwaukee, WI, USA). The reference steroids were from Steraloids (Wilton, NY, USA) and from Research Plus (Bayonne, NJ, USA).

2.2. Urine samples

Eight healthy male volunteers, 19 to 52 years old, participated in the study relative to the determination of the endogenous production of 19-NA. Urine samples were taken at different moments of the day (7, 11, 17 and 23 h, February 1998). Six urine samples coming from a healthy male volunteer were collected over a period of 3 months. Three healthy male volunteers, 22 to 30 years old, gave their urine before and after a prolonged intense effort. One healthy male volunteer received a 10 μg oral dose of nandrolone. After collection, urine samples were stored below −18°C.

2.3. Extraction and purification procedure [46–49]

After addition of internal standard (4-chlorotestosterone) and 2 ml of acetate buffer (pH 5.2, 0.2 *M*), 10 ml of urine was hydrolysed (at least 15 h, 52°C, pH 5.2) with 100 μl of *Helix pomatia* juice (β-glucuronidase 10^5 Fischman U/ml and sulfatase 10^6 Roy U/ml). The urinary extract was centrifuged at 2000 *g* for at least 10 min. The sample was then applied to a C18 column previously conditioned with 10 ml of methanol and 10 ml of ultrapure water. The column was washed with 10 ml of ultrapure water and 10 ml of hexane. Steroid analytes were eluted with 5 ml of methanol–ethyl acetate (30:70, v/v). The eluate was washed twice with 2 ml of 1 *M* sodium hydroxide. The combined organic layers were evaporated to dryness under a gentle stream of nitrogen. The dry residue was dissolved in 1 ml of 1,1,1-trichloroethane–ethyl acetate (80:20, v/v) and introduced into an ultrasonic bath. The resulting extract was then applied to a silica gel column (I.D.: 1 cm; length: 8 cm) conditioned with 1,1,1-trichloroethane–ethyl acetate (80:20, v/v). A 17-ml aliquot of this mixture was used to wash the stationary phase. The analytes were eluted with 13 ml of 1,1,1-tri-

chloroethane–ethyl acetate (20:80, v/v). The eluate was evaporated to dryness under reduced pressure. The residue was dissolved in 30 μl of hexane–propan-2-ol (90:10, v/v) and then purified on a NH_2 HPLC column (Nucleosil Macherey Nagel, Habrot, France) (25×0.4 cm I.D.) coupled to a fraction collector (Gilson 203, Middleton, WI, USA). The mobile phase composition was hexane–propan-2-ol–THF (90:2.5:7.5, v/v/v). The isocratic mode was applied during 15 min; the proportion of propan-2-ol was increased during the ten following minutes until reaching 50% of the mobile phase composition. The flow-rate was 2 ml/min. Eluate fractions (2 ml) were collected at 1-min intervals. After addition of norgestrel (external standard), collected fractions were evaporated to dryness before derivatization.

The immuno-affinity columns used for the clean-up of some growth promoters (stilbenes (ex: DES), resorcylic acids (ex: zeranol) and 19-norsteroids (ex: nandrolone)) were conditioned with 5 ml of ultrapure water and 5 ml of acetate buffer (pH 5.2, 0.2 *M*). After extraction on a SPE C18, the urine residue was dissolved in acetate buffer (pH 5.2, 0.2 *M*) and introduced in the column. The sample is let 5 min in contact with the antibody gel. After elution, the column was washed twice with acetate buffer (pH 5.2, 0.2 *M*) and eluted with 3 ml of acetone.

2.4. Derivatization

The dry residue was derivatized according to two procedures: (reaction A) 60 min at 80°C with MSTFA–TMIS–DTE (1000:5:5, v/v/w) or (reaction B) 75 min at 80°C with MTBSTFA–TBDMSCl–NH_4I–CH_3CN–C_5H_5N (900:9:5:25:75, v/v/w/v/v) [50].

2.5. GC–MS analysis

The quadrupole MS used was a 5989A model coupled to a 5890 gas chromatograph both from Hewlett-Packard (Palto Alto, CA, USA). The magnetic MS was a reverse geometry double focusing instrument SX102A (Jeol, Tokyo, Japan) coupled to a HP-5890 GC. The transfer line temperature was set at 280°C and the split/splitless injector was maintained at 250°C (time of splitless set at 1 min). The column used was a HP-1 (Hewlett-Packard) (30 m×0.25 mm I.D., film thickness 0.25 μm). The GC parameters used were 120°C (2 min), 15°C/min until 250°C (0 min) then 5°C/min until 300°C (5 min). Helium (N55) was used as carrier gas at 1 ml/min. The electronic beam energy was set at 70 eV in the electronic impact (EI) mode.

3. Results and discussion

3.1. Derivatization

Two modes of derivatization were selected because of their sensitivity and specificity (at least four diagnostic ions). The more classical one, i.e. the one used by numerous anti-doping laboratories (IOC accredited), is the reaction involving the MSTFA–TMIS–DTE mixture. This reagent leads to the formation of the 3-TMS-ether-17-TMS-enol derivatives of 19-NA and 19-NE. The electron impact mass spectra of the two isomers are characterised by a prominent molecular ion (*m/z* 420), an intense $(M\text{-}CH_3)^+$ (*m/z* 405), and two diagnostic ions of minor abundances, the $(M\text{-}CH_3\text{-}TMSOH)^+$ and the $(M\text{-}CH_3\text{-}2TMSOH)^+$, respectively at *m/z* 315 and 225 (Fig. 2). Because of the high sensitivity of the *m/z*

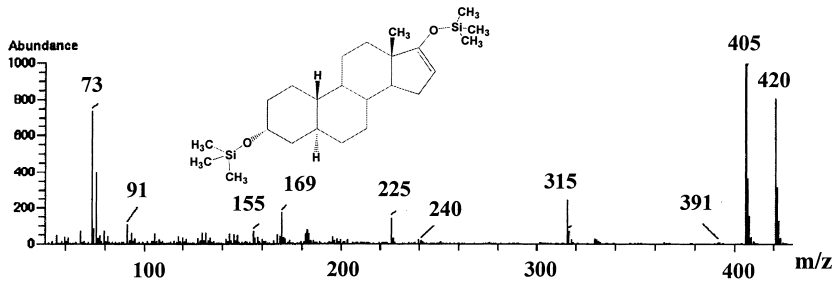

Fig. 2. TMS derivatization. EI mass spectrum of 3-TMS-ether-17-TMS-enol derivative of 19-norandrosterone.

405 signal, this ion was the more suitable for detection and quantification purposes.

On the other hand, the reagent B [50] authorised the synthesis of the 3-*tert*-butyldimethylsilyl-ether-17-*tert*-butyldimethylsilyl-enol forms of the two nandrolone metabolites. Even if this derivative is a little bit less sensitive than the first described, it permitted to obtain more ions of high masses under EI conditions. It authorised a more important retention time, so a more specific mass chromatogram as well. The mass spectra are characterised by the presence of two weak ions at m/z 504 ($M^{+\cdot}$) and 489 $(M\text{-}CH_3)^+$, the base peak at m/z 447 corresponding to the loss of a *tert*-butyl group and two intense ions at m/z 391 and 371 (Fig. 3). This derivative was used for the unambiguous identification of the steroid analytes, according to the 93/256/EEC-decision [51].

3.2. Extraction and purification

The strategy adopted by the different analytical laboratories is different. Some approaches focus on fast extraction procedures in order to be able to treat-large number of samples. In this case, the analytical method must include very specific measurements, like high-resolution mass spectrometry or tandem MS (MS^2 thanks to triple quadrupole mass spectrometers or MS^n with ion trap instruments). The second approach is to realise the best possible purification in order to reduce false positive risk and overestimation during quantification exercises, but the drawback is the more time consuming of the sample preparation. Nevertheless, we have chosen this strategy. The comparison of more or less sophisticated purification procedures is shown in Fig. 4 [52]. One can judge the differences in terms of interferences and the consequent relative ability to interpret the ion chromatograms. Of course, with a single quadrupole mass spectrometer, a single SPE as well as the combination of a two SPE (a C18 and a SiOH stationary phase) and an alkaline LLE, are not sufficient to guarantee efficient diagnostic. On the other hand, an immunoaffinity clean-up and better, a semi-preparative HPLC, give a very 'clean' ion chromatogram without any interference. The system adopted in this study was the HPLC system, with a specific aminopropyle stationary phase, and a mixture of easily evaporable organic solvents as a mobile phase. Fraction collections authorised very effective clean-up of 19-NA and 19-NE and also separation of the two metabolites, one from the other. In Fig. 5, the different interference signals around the expected retention time of 19-NA (down), are definitely removed (up). The limit of detection of the method was estimated using 0.1 μg/l (ppb) spiked urine. The calculated limit of detection was 0.02 ng/ml on the m/z 405 base peak, signal to noise ratio better than three.

3.3. Method evaluation

The efficiency of the deconjugation step is difficult to evaluate because of the lack of reference standards (sulfo and glucurono conjugates of 19-NA and 19-NE) and the never totally reproducible in vivo conditions. Consequently, in order to guarantee the applicability of the method on real samples, especially the efficiency of the deconjugation step, we have worked on: pregnant woman, urine known to contain

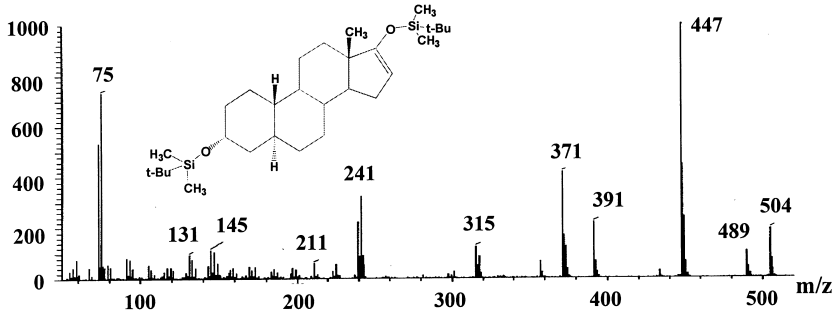

Fig. 3. TBDMS derivatization. EI mass spectrum of 3-*tert*-butyldimethylsilyl-ether-17-*tert*-butyldimethylsilyl-enol derivative of 19-norandrosterone.

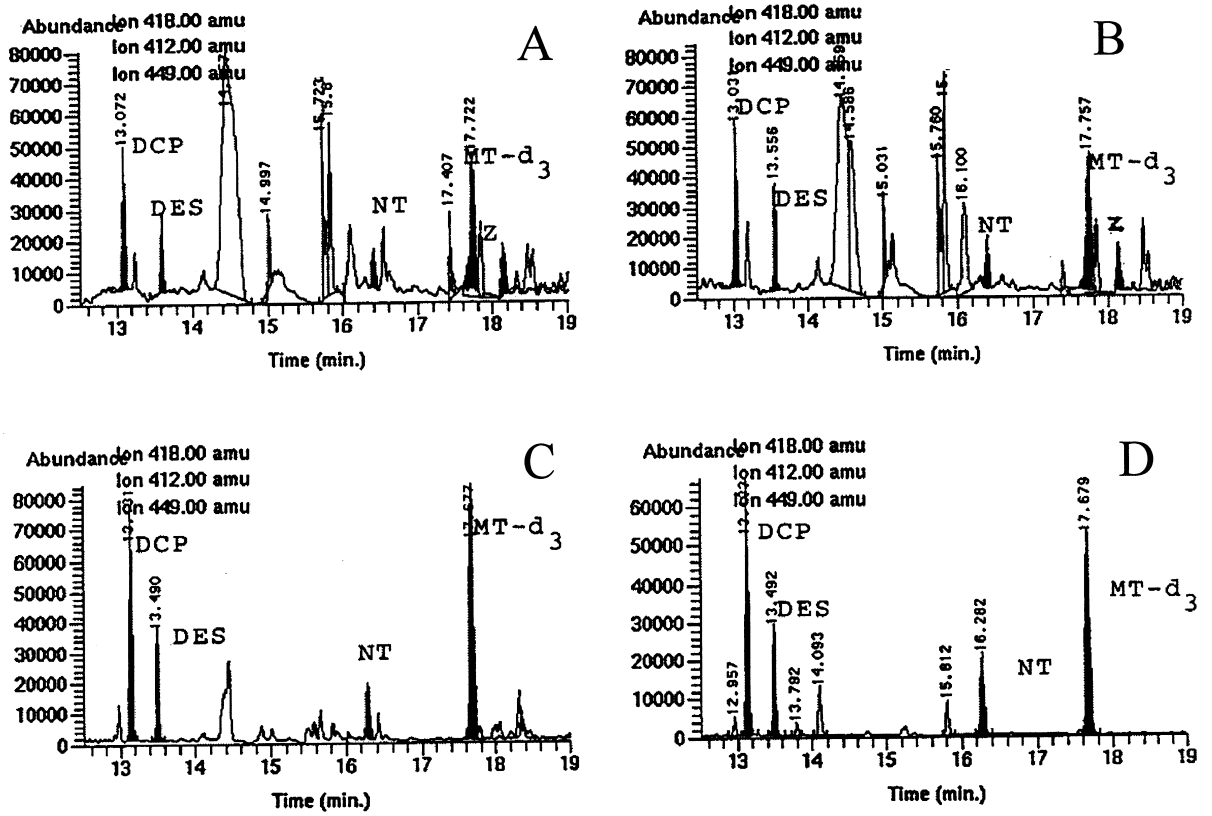

Fig. 4. Comparison of different clean-up procedures in terms of purification efficiency. Ion chromatograms of urine extracts spiked with 2 ng/ml of diethylstilbestrol (DES), 19-nortestosterone (NT) (EI ionization, SIM measurement, internal standard: MT-d_3 (d_3-methyltestosterone), external standard: DCP (dichlorophene)) (A: C18/LLE; B: C18/LLE/SiOH; C: C18/LLE/IAC; D: C18/LLE/SiOH/HPLC).

endogenous 19-NA and 19-NE (Fig. 6, middle) ; urine taken from an adult male who has received an oral administration of 19-NT (Fig. 6, right).

Even if the two expected metabolites were observed in the two samples, the real efficiency of the crude extract of *Helix pomatia* was difficult to evaluate. We have verified the absence of the side effects of this enzymatic juice on 19-NA and 19-NE, adding the enzyme preparation on the two standards and verifying the non-degradation and the non-transformation via an interfering isomerase or dehydrogenase.

However, interlaboratory comparison on positive urine (measures realised in LDH/LNR, Nantes and LAB, Châtenay-Malabry, France; results not yet published) has shown: a comparable efficiency of *Helix pomatia* and β-glucuronidase from *E. Coli* enzymes for the deconjugation of 19-NA (measured concentrations not significantly different); a better efficiency of *E. Coli* β-glucuronidase compared to the *Helix pomatia* enzyme preparation in the 19-NE conjugate hydrolysis.

Complementary studies have been initiated.

3.4. Detection of 19-norandrosterone

The urine of eight volunteer adult males working in the laboratory was taken at different moments of the day (7, 11, 17 and 23 h). On a total of 32 urine samples, 18 (more than 50%) showed a suspect signal at the expected retention time of 19-NA (Table 1); no 19-NE was observed above the detection limit (LOD) of the method (0.02 ng/ml) (Fig. 7).

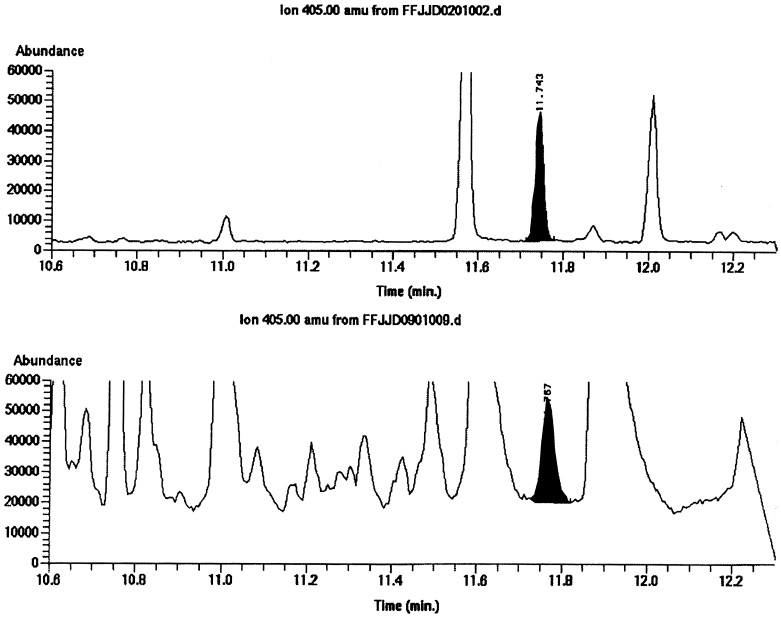

Fig. 5. Clean-up efficiency of a semi-preparative HPLC. Ion chromatograms (m/z 405) relative to 19-NE spiked urine with (up) or without (down) semi-preparative HPLC.

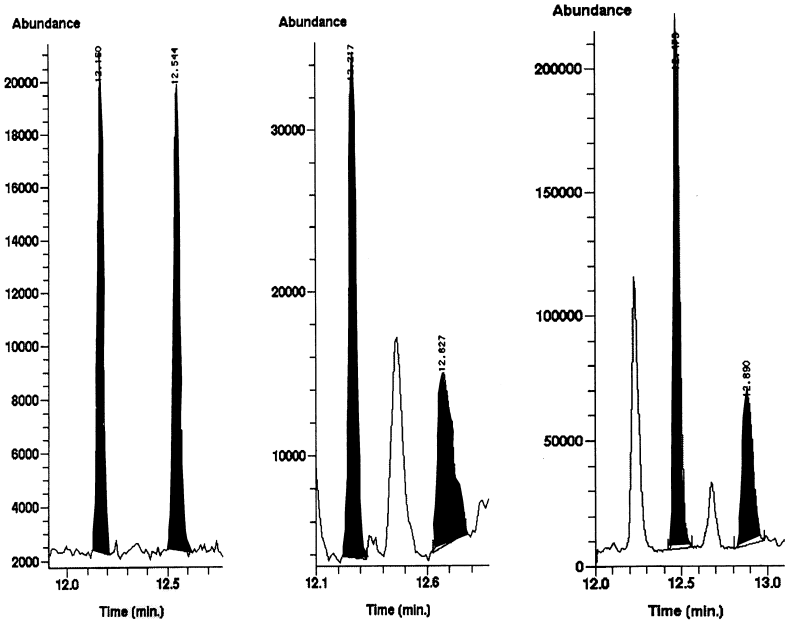

Fig. 6. Method of evaluation on incurred samples. m/z 405 ion chromatograms corresponding to a 0.2 ng injected 19-NE and 19-NA standard (left), to urine sample collected from a pregnant women (5th month of pregnancy) (middle) and to urine 24 h after an oral administration of nandrolone to a male adult (right).

Table 1
Suspected 19-NA in 32 samples (0: not detected, +: concentrations between LOD and 0.3 ng/ml, ++: concentrations between 0.2 ng/ml and 0.6 ng/ml)

Individual	Sex/Age	7 h	11 h	17 h	23 h
6	M/19	+	0	0	0
8	M/20	+	++	++	++
5	M/21	+	0	0	0
3	M/24	++	+	+	++
1	M/30	+	0	++	0
2	M/46	++	+	+	0
7	M/51	0	0	+	+
4	M/52	0	0	+	0

3.5. Identification of 19-norandrosterone

3.5.1. Co-chromatography

In order to confirm the identity of the observed analyte, we have used a co-chromatography technique. For this purpose, we have added increasing concentrations of TMS-derivatized 19-NA and 19-NE in the previous derivatized extract. In Fig. 8 are shown the resulting mass chromatograms. When 1 ng was added in the vial (Fig. 8B), the height of the suspected analyte signal increased without being divided; the peak symmetry remained perfectly Gaussian. The addition of 19-NA and 19-NE (Fig. 8C) confirmed this first observation and moreover permitted to confirm the absence of 19-NE at the LOD.

3.5.2. Semi-preparative HPLC and complementary derivatization

The theoretical fractions of 19-NA and 19-NE were collected after HPLC separation. Fractions 4 and 5 were analysed in GC–MS. Each fraction was divided into two equal volumes, each one being derivatised according to the two procedures described earlier. 19-NA was detected in the 4th fraction as expected. The TMS and TBDMS derivatized extracts are respectively in Figs. 9 and 10. On these figures are shown the diagnostic ion chromatograms m/z 420, 405 and 315 (up) and the SIM mass spectrum (down) of a 19-NA spiked sample (left) and of a volunteer urine (right). The TBDMS derivatization brought a new retention time, complementary diagnostic ions characterised by different ratios compared to the TMS one. The 5th fraction did not contain 19-NE.

This very selective purification as well as the

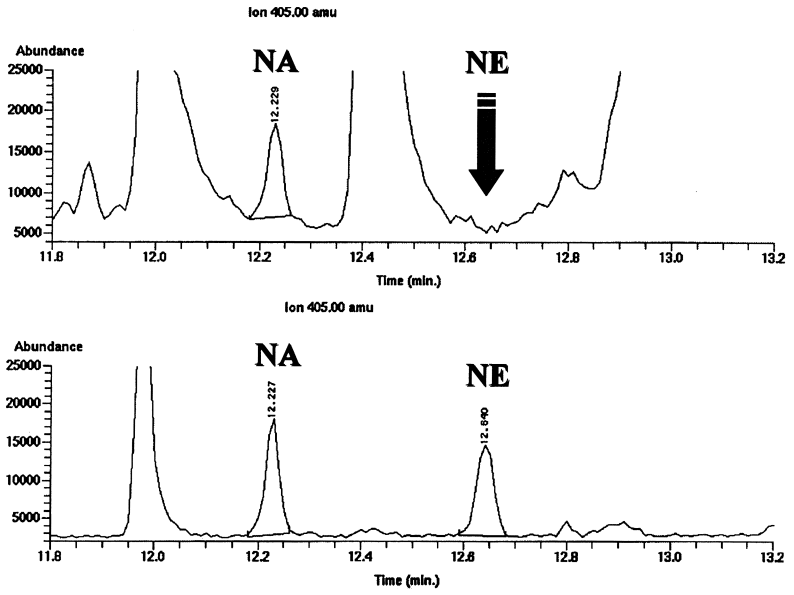

Fig. 7. Detection of endogenous 19-norandrosterone. Ion chromatograms (m/z 405) corresponding to a healthy volunteer urine (up) and to a 0.5 ng/ml 19-NA and 19-NE spiked sample (down).

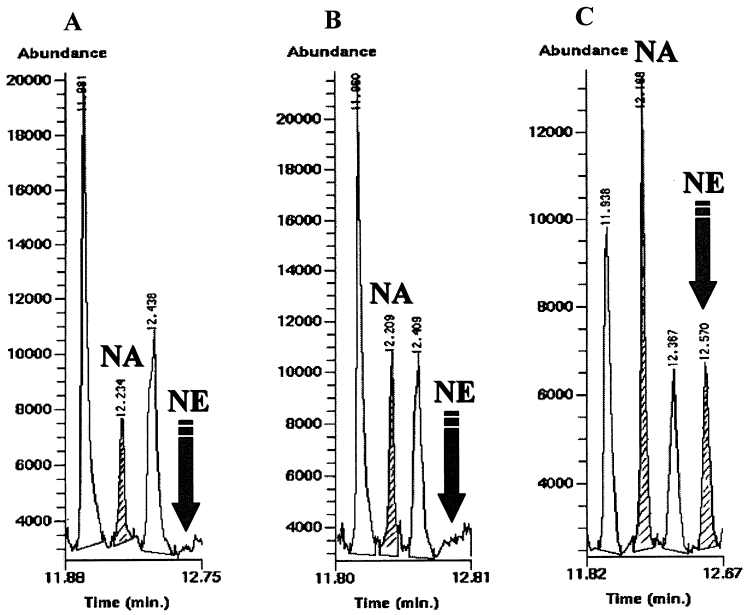

Fig. 8. Identification of 19-NA by co-chromatography. Ion chromatograms (m/z 405) corresponding to an healthy volunteer urine (A), spiked with 1 ng of TMS-derivatised 19-NA (B) and 5 ng of TMS-derivatised 19-NA and 19-NE.

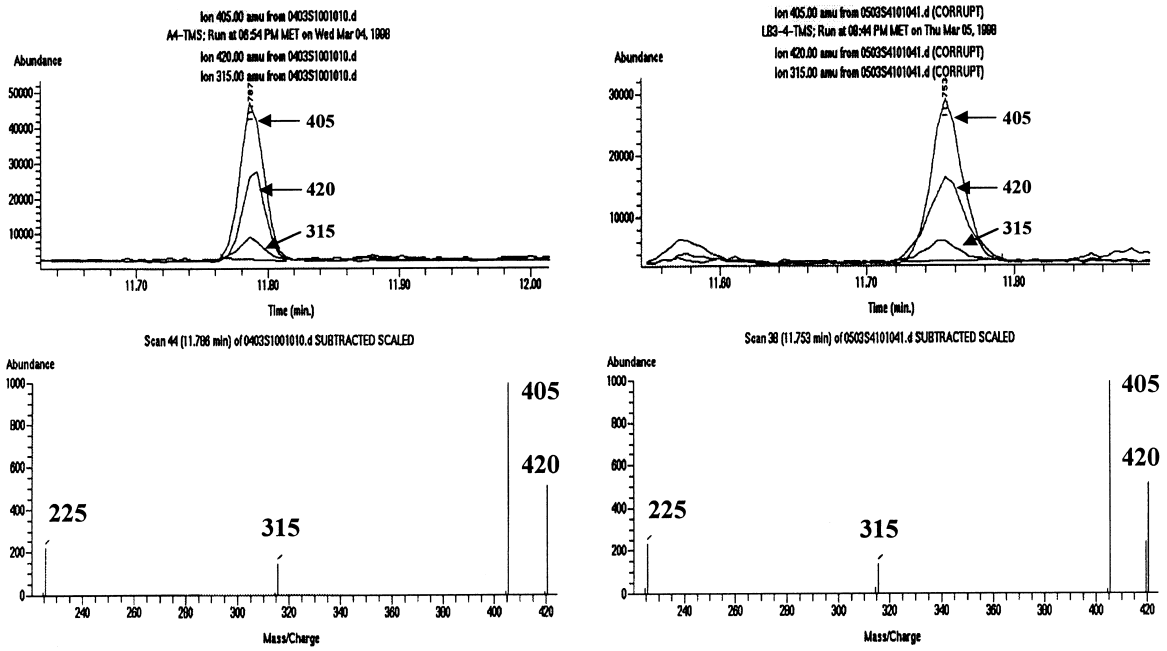

Fig. 9. Identification of 19-NA by semi-preparative HPLC and complementary derivatization. Ion chromatograms corresponding to a spiked urine (left) and to a healthy volunteer urine (right) (TMS derivatization, EI ionization, SIM and low-resolution measurement). 19-NA concentration was measured at 0.4 ng/ml.

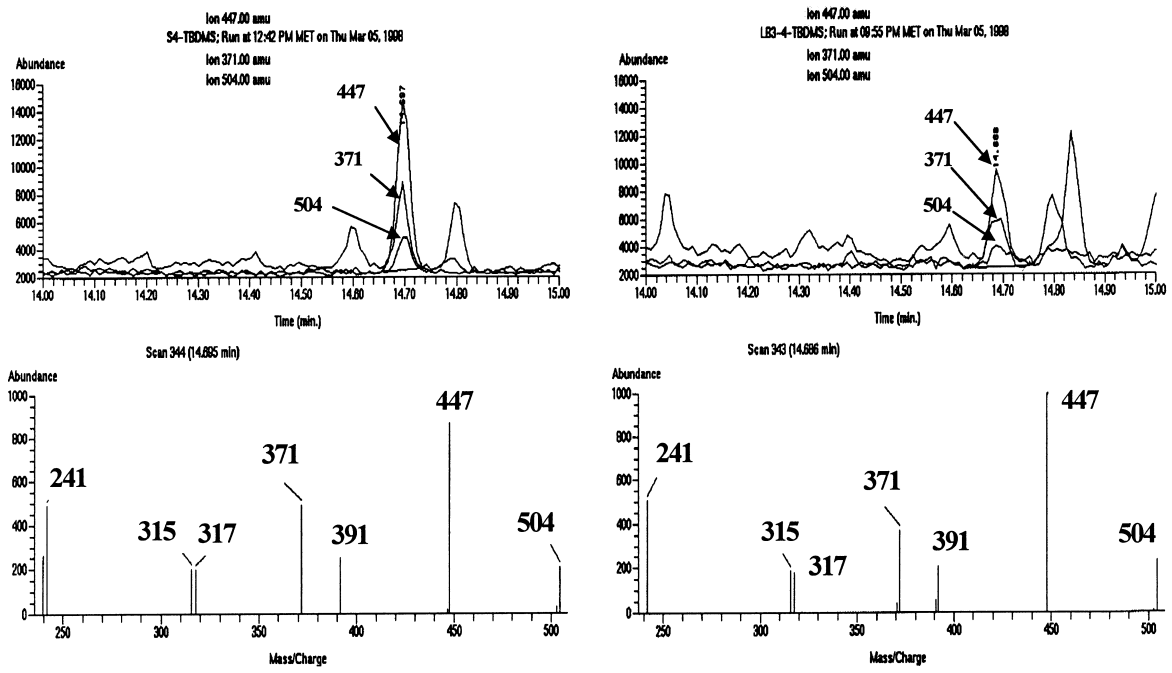

Fig. 10. Identification of 19-NA by semi-preparative HPLC and complementary derivatization. Ion chromatograms corresponding to a spiked urine (left) and to a healthy male volunteer urine (right) (TBDMS derivatization, EI ionization, SIM and low resolution measurement). 19-NA concentration was measured at 0.4 ng/ml.

complementary derivatization confirmed once again the identity of the suspected analyte.

3.5.3. Acquisition in the HR-SIM mode (R= 10 000)

The structural identification was definitely realised by high-resolution mass spectrometry measurements at a resolution of 10 000 (10% valley definition) after a HPLC fractionation. Mass chromatograms corresponding to a blank urine (left) and to urine taken from a healthy volunteer (right) are shown in Fig. 11. The exact masses of four diagnostic ions were recorded for the two metabolites (420.2880, 405.2645, 315.2144 and 225.1643). The four signals were observed at the expected retention time for 19-NA and in the good ratios (±10%) according to the analytical criteria of the 93/256/EEC directive [51]. Acting as a very specific clean-up, this measurement method permitted the unambiguous identification of 19-NA in urine of non-treated adult male persons.

3.6. Estimation of physiological 19-NA concentration

Because endogenous 19-NA was unambiguously found and because a decision limit of 2 ng/ml is applied for some molecules like nandrolone and boldenone metabolites [53], it was necessary to establish on the available samples, the physiological concentrations of 19-NA. For this purpose, we have quantified the 19-NA concentration in the 18 mentioned urine samples (cf. section 3.4) using the method previously described (semi-preparative HPLC, GC–MS, EI, SIM, 4-chlorotestosterone as internal standard). The calibration curve was based on four extracted spiked urine (0, 0.1, 0.3 and 0.5 ng/ml) and the m/z 405 signal height versus concentration (Fig. 12). The measured concentration varied between the method detection limit 0.02 and 0.6 ng/ml (Table 1). The 19-NA concentrations in the urines of the analysed serie were often between 0.1 and 0.3 ng/ml. No correlation could be done between the concentrations and the sampling hour

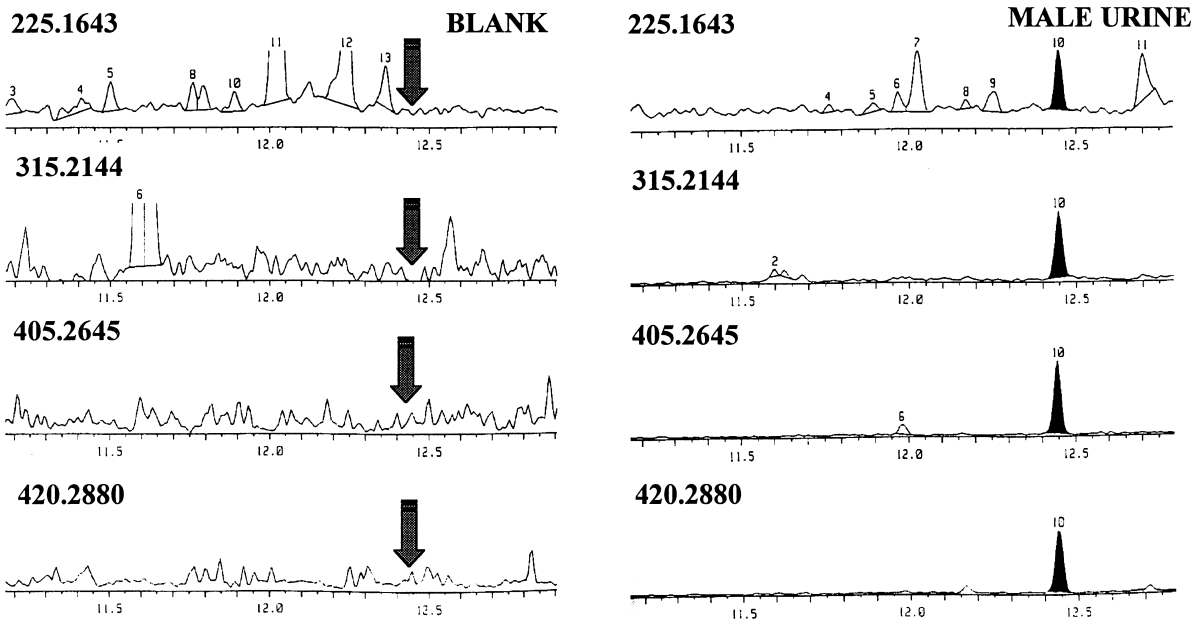

Fig. 11. Identification of 19-NA by high resolution mass spectrometry measurement. Ion chromatograms (from up to down m/z 225.1643, 315.2144, 405.2645 and 420.2880) corresponding to a blank urine (left) and to a healthy volunteer urine (right). 19-NA concentration was measured at 0.4 ng/ml.

nor the age of the volunteers, excepting the fact that 19-NA was almost always detected in the first urine of the day. We have extended the follow-up of 8 persons on one day, to one individual on 3 months. This subject was a male adult of 26 years of age, sportsman of international rank. The data relative to the analysed urine, i.e. the dates, m/z 405 chromatograms and the respective measured concentrations, are shown in Fig. 13. The values found for 19-NA varied between 0.06 and 0.47 ng/ml, with most of the concentrations included between 0.1 and 0.2 ng/ml.

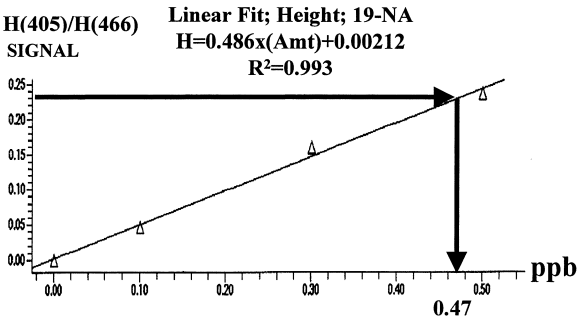

Fig. 12. Quantification of 19-norandrosterone. Calibration curve with on the y-axis the ratio between m/z 405 of 19-NA and m/z 466 of the internal standard 4-chlorotestosterone, and on the x-axis the 19-NA concentration in ng/ml.

3.7. Effort consequences

The relative high concentrations of 19-NA found sometimes in urine of random fellows (0.5 to 0.6 ng/ml), are not so far from the action level (2 ng/ml) applied in the IOC laboratories [53]. One can imagine that before or during special sport events, a changing of the basal concentrations can occur (because of the stress of competition or the effort itself), so that an endogenous concentration can be interpreted as an exogenous administration with all the consequences that follow. The urine of a soccer player (male, 22 years old, 72 kg, 1m80) was taken before and after a football match. The relative m/z 405 chromatograms are shown in Fig. 14. The intensity of the 19-NA signal was increased by a factor of three after the effort. This phenomenon, probably due to the concentration of the urine (linked to the dehydration during the effort), could

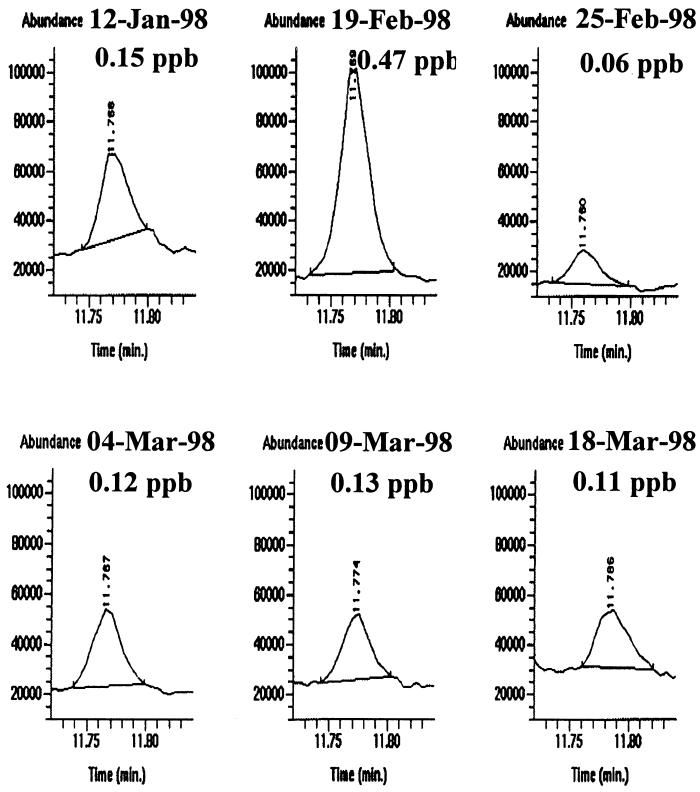

Fig. 13. Quantification of 19-norandrosterone in one male person. The m/z 405 ion chromatograms corresponding to the urine sample of a sportsman sampled during 2 months. The measured concentrations are indicated at the apex of each signal.

generate false positive results on people having a basal 19-NA level above 0.5 ng/ml. An epidemiological study must be realised on a large number of people in order to determine physiological values for 19-NA in male adult urine and the consequences of prolonged effort coupled to the competition stress on this population. At this moment, the IOC decision level would be probably thrown back into question.

3.8. Tendentious urine profiles

Two urine samples that were considered as suspect during official controls were analysed in our lab according to the described method. The profiles are shown in Fig. 15. One can observe the atypical profiles compared to the one shown before. Unlike the endogenous (Fig. 7) or the exogenous (Fig. 6, right) 19-NA and 19-NE urinary profiles, here the ratios between 19-NA and 19-NE were very uncommon, since 19-NE was at least as abundant as 19-NA. Moreover, the concentrations found in these urine samples were for the first individual 5.6 ($\pm$0.6) ng and 8.2 ($\pm$1.0) ng, respectively for 19-NA and 19-NE, and 4.3 ($\pm$0.5) ng and 8.7 ($\pm$1.1) ng for the second individual (Table 2). 19-NA was ten-fold more concentrated in these two urine samples than the maximum values observed in the 'healthy volunteer population' tested for in our lab. The level of urinary 19-NE was considerably overstepping the concentrations found in the healthy volunteer urine.

However, that may be, further investigations have to be done in the presence of such atypical urine sample; some precautions have to be taken before giving any conclusion about a possible 19-NT positivity. The 19-NA and 19-NE origin from synthetic androgenic steroids like 19-norandrostenedione or 19-norandrostenediol should also be investigated.

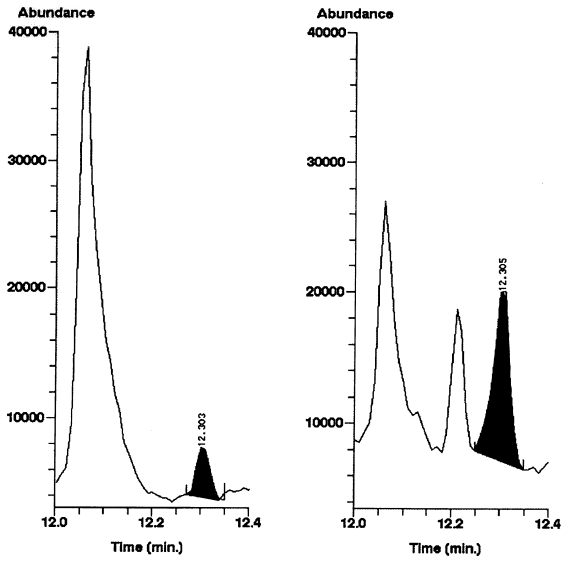

Fig. 14. Effort consequences. The m/z 405 ion chromatograms corresponding to the urine sample of a sportsman sampled before (left) and after (right) a soccer competition.

4. Conclusion

A specific and sensitive analytical method has been developed in order to detect and to identify low quantities of 19-NA and 19-NE, two human metabolites of a well-known anabolic steroid, 19-nortestosterone. 19-NA was detected and identified in the urine of healthy volunteers (18 on 32 samples) not treated by 19-nortestosterone. The concentrations found in the 18 urine samples varied between 0.02 and 0.6 ng/ml. The 19-NA concentrations in the urine of the analysed serie, levels which could be called 'endogenous noise', were between 0.1 and 0.3 ng/ml. This follow-up on one day was extended on 3 months. The studied subject excreted 19-NA at concentrations between 0.06 and 0.47 ng/ml urine with most of the values included between 0.1 and 0.2 ng/ml. Several hypotheses have been done concerning the possible origin of this molecule, but its natural origin was never discussed before in human male adults. The production of this molecule by human organisms was envisaged after consumption of growth promoter contaminated meat. Some publications related yet the possibility of such interferences in doping control [54–56]. Indeed, nandrolone and its esters have been used since 1990 as growth factors in meat producing animals. But examining the pharmacokinetics of 19-NT in man [57–59] and making a rapid calculation between nandrolone residue levels in meat of treated animals (10 to 100 pg/g order) and measured residue concentrations in human urine (100 to 600 pg/ml order), one can conclude that the swallowed meat quantity must be in the kg order! Furthermore, the finding of classical anabolic steroid (as 19-nortestosterone) treatment for fattening purposes is decreasing over the last several years according to the statistics of the EU National Reference Laboratories in charge of the control. This observation is once again not in favour of the contamination hypothesis. We also made the assumption that the consumption of non-castrated pig livers (in pâté for instance) could be a potential source of exogenous supply. Indeed, the endogenous production of 17β-19-nortestosterone by intact young boars was demonstrated before [32–35]. Relative high levels were found in different biological matrices; the mean and highest concentrations measured by van Ginkel et al. [33] were 1 and 13 ng/g in muscle and 23 and 200 ng/g in liver, respectively. Even if highly improbable, this track can not be definitely eliminated, the non-castrated pigs being transformed and incorporated in delicatessens.

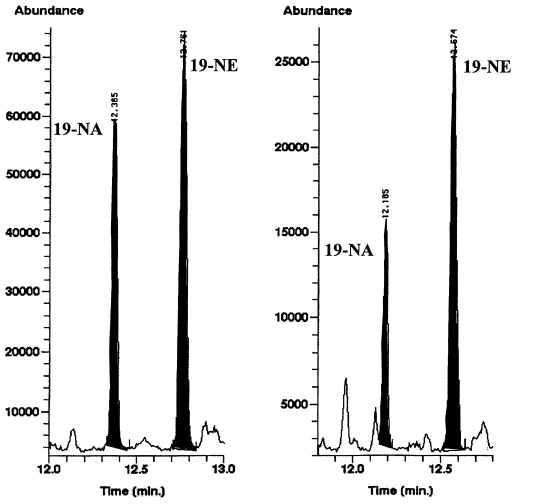

Fig. 15. Urinary profiles always submitted to some interrogations. The m/z 405 ion chromatograms corresponding to the urine sample of two sportsmen (left: judoka; right: football player).

The consequences of an intense effort (soccer competition) have also been studied; the 19-NA

Table 2
19-NA and 19-NE concentrations in urine considered as suspect during official controls (in ng/ml)

Individual 1			Individual 2		
Measures	19-NA	19-NE	Measures	19-NA	19-NE
1	5.2	7.6	1	3.6	7.2
2	4.8	6.8	2	4.1	7.9
3	5.9	8.3	3	4.1	8.5
4	6.1	8.7	4	5.0	10.1
5	5.2	8.3	5	4.4	9.6
6	6.5	9.6	6	4.3	9.0
Mean value	6.5	8.2	Mean value	4.3	8.7
Standard deviation	0.6	1.0	Standard deviation	0.5	1.1

concentrations found, proved to be increased by a factor of three after the effort. One can imagine that in certain conditions, i.e. very long and intense exercises (marathon runner, football player, racing cyclist, etc.) or dehydration periods before some specific competitions (loss in weight in some sports due to weight categories (judoka, boxer, etc.) or for others reasons (jockey)), the 19-NA apparent concentration in urine can increase by factors higher than those observed in our experience. Consequently, individuals whose 19-NA basal level would be slightly inferior to 1 ng/ml, could have in some conditions (pronounced dehydration for example) this value reach 2 ng/ml, indeed even exceed this action level. As nobody can exclude this hypothesis, further experiments have to be done on a large scale, on athletes evolving in different sport disciplines.

What could be done for the moment in the antidoping control field?

Physiological levels have to be determined on a large population in order to determine an official action level. The two main inconveniences will be the time needed to establish these values and the possible existence of particular individuals who can excrete 19-NA and 19-NE at higher concentrations.

Some laboratories apply some internal criteria, based on their own experience. Dr. Schänzer (Deutsche Sporthochschule Köln), mentioned that his predecessor, Prof. Dr. Donike, did not consider low norandrosterone levels in urine as an offence to doping [60]. He did not exclude in man that under special circumstances, 19-NA can be produced endogenously and excreted into urine in low concentrations. Dr. Donike considered a threshold value based on the ratio of 19-NA to endogenous androsterone and etiocholanolone [60]. In spite of its empiricism, this decision method, applied in one of the reference IOC accredited laboratories, could be an opening for further international applied and recognised criteria.

Other possibilities could consist of studying the endogenous 19-NA to 19-NE relative quantities and to determine a discriminatory ratio to distinguish the endogenous presence from the exogenous one. Some urine measurements on doped individuals showed a ratio between 19-NA and 19-NE comprised between 3:1 and 5:1; Dr. Schänzer related a ratio of 72:28 [19]. Another approach would be to study the sulfo and glucurono forms of 19-NA and 19-NE, focusing on the relative concentrations of the phase II metabolites according to their exo- or endogenous origins. In the same scope, it could be interesting to explore the behaviour of another 19-nortestosterone metabolite in comparison with 19-NA and 19-NE when 19-nortestosterone is administered or not.

Another way to be investigated in the scope of the testosterone abuse in sport [61–64], in equine [65] or in farm animals [66], would be to develop analytical methods based on the combination of gas chromatography and isotope ratio mass spectrometry via a combustion furnace (GC–C–IRMS). Indeed, endogenous 19-NA and 19-NE metabolites possess a given $^{13}C/^{12}C$ ratio. The $^{13}C/^{12}C$ ratio of synthesised steroids are generally lower than those of the corresponding endogenous ones [65]. Consequently, the $^{13}C/^{12}C$ ratio of urinary 19-NA and 19-NE should

decrease when 19-NT is exogenously administered. Nevertheless, because of the trace amounts of 19-NA and 19-NE in urine compared to the corresponding metabolites of testosterone, one of the main problems for the time being and the near future will be the lack of sensitivity of the GC–C–IRMS instruments, even some noticeable overhang on the last months can be underlined.

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