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IDENTIFICATION OF ENDOGENOUS 19-NORANDROSTERONE IN HUMAN URINE

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Abstract

Until now, presence demonstration of 19-norandrosterone (19-NA: 3α-hydroxy-5α-estran-17-one) and 19-noretiocholanolone (19-NE: 3α-hydroxy-5β-estran-17-one) signed undoubtedly exogenous administration of nandrolone (19-nortestosterone: 17β-hydroxyestr-4-en-3-one). Between 1997 and 1999, cases of athletes popularised through the media which urines contained nandrolone metabolites generated some doubts about criteria of positivity. The possible production of the target metabolites, 19-NA and 19-NE, in adult male was suggested. Some studies were realised on different persons known to be non-treated with nandrolone. Two main objectives were defined: the unambiguous identification of potential endogenous 19-norandrosterone and 19-noretiocholanolone, and determination in several individuals of the common concentrations of these two metabolites. Gas chromatography coupled to mass spectrometry (quadrupole and magnetic instruments) was used to detect, identify and quantify the suspected signals. Two types of derivatisation, semi-preparative HPLC as well as co-chromatography proved unambiguously the identity of 19-NA in more than 50 % of the analysed urines (n=40) at concentrations between 0.02 and 0.60 ng/ml.

Introduction

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.* [1]. Two main metabolites were isolated: 3α-hydroxy-5α-estran-17-one (19-NA) and 3α-hydroxy-5β-estran-17-one (19-NE) (Fig. 1). In the antidoping field, the illegal administration of 19-NT is controlled on the basis of the presence of these two metabolites [2-6], which are present in the ratio 70:30 respectively for 5α and 5β metabolites [6]. Nevertheless, with the introduction of

more specific and sensitive methods (quadrupole MS/MS, ion trap MS^n, and double focusing instruments authorising high resolution measurements) some steroids were proved to be endogenously synthesised. It was the case of 17β-nandrolone in stallions [7-10] and in equine follicular fluid [11-12]. In the same period, natural production of 19-nortestosterone and 19-norandrostenedione in the follicular fluid and plasma of pregnant women was observed as well [11,13]. Later on, 17β-nandrolone was proved to be produced by intact boar [14-17], epinandrolone by pregnant cows, newborn calf [18-24] and pregnant sheep [25]. In horse doping analysis and in the control of anabolic steroid residues in slaughter animals, appropriate measures were taken to exclude false positive results. In human doping analysis, some works have been done related to the possible natural presence of 19-norandrosterone and 19-noretiocholanolone in human male urine [26-29]. Because of the ultra-trace level (<100 ppt), development of specific and sensitive GC-MS method was necessary.

Experimental [30]

Most of the reagents and solvents were of analytical grade quality and provided by Merck (Darmstadt, Germany) and Solvants Documentation Synthesis (SDS, Peypin, France). The derivatisation reagents N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), N-methyl-N-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA), *tert*-butyldimethylchloro-silane (TBDMCS) and trimethyliodosilane (TMIS) were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTT) was from Aldrich (Milwaukee, WI, USA). *Helix pomatia* juice was from Biosepra (Villeneuve la Garenne, France) and β-glucuronidase (*E. Coli*) was provided by Boehringer (St. Quentin Fallavier, France). Eight healthy men volunteers, 19-52 years old, participated in the study. After collection, urine samples were stored below - 18°C. Ten ml of urine were hydrolysed (at least 15 h, 52°C, pH 5.2) with 100 µl of *Helix pomatia* juice (β-glucuronidase 10^5 Fischman U ml^{-1} and sulfatase 10^6 Roy U ml^{-1}) or 100 µl of β-glucuronidase from *E. coli*. The sample was then applied on a C18 column. Steroid analytes were eluted with 5 ml of methanol-ethyl acetate (30:70, v/v). The eluate was washed twice with 2 ml of 1M sodium hydroxide. After evaporation, the dry residue was taken back in 1,1,1-trichloroethane-ethyl acetate (80:20, v/v) and applied to a silica gel column (i.d.: 1 cm; length: 8 cm). After washing the stationary phase with 1,1,1-trichloroethane-ethyl acetate (80:20, v/v) analytes were eluted with 1,1,1-trichloroethane-ethyl acetate (20:80, v/v). After evaporation, the dry residue was restored in hexane-propan-2-ol (90:10, v/v) and then purified

on a NH_2 HPLC column (Nucleosil Macherey Nagel, Habrot, France) (25 x 0.4 cm i.d.) which mobile phase was hexane-propan-2-ol-THF (90:2.5:7.5, v/v/v). The dry residue was derivatised 40 min at 60°C with MSTFA-TMIS-DTT (1000:5:5, v/v/w). Quadrupole MS used were a 5989A or a 5973 respectively coupled to a 5890 and 6890 gas chromatographs both from Hewlett Packard (Palo Alto, CA, USA). The magnetic MS was a reverse geometry double focusing instrument SX102A (Jeol, Tokyo, Japan) coupled to a HP-5890 GC.

Results and discussion

Derivatisation

Two modes of derivatisation were selected because of their sensitivity and specificity (at least four diagnostic ions). MSTFA-TMIS-DTT 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) suitable for detection and quantification purposes, and two diagnostic ions of minor abundance at m/z 315 and 225 (figure 1a). Mass spectra of this derivative in the positive and negative chemical ionisation modes are shown on figure 1b and 1c. The PCI mass spectrum is characterised by one intense quasi molecular ion $(M+H)^+$, three diagnostic ions $(M\text{-}CH_3)^+$, $(M+H\text{-}TMSOH)^+$ and $(M\text{-}CH_3\text{-}TMSOH)^+$. The NCI mass spectrum is characterised by a weak quasi-molecular ion $(M-H)^-$, two intense peaks corresponding to $(M\text{-}TMS)^-$ and $(M\text{-}2TMS)^-$. Unfortunately, and contrary to electronic impact conditions, these chemical ionisation modes were incompatible with the detection limit we wanted to reach. Reagent B [32] authorised the synthesis of the 3-*tert*-butyldimethylsilyl-ether-17-*tert*-butyldimethylsilyl-enol forms of 19-NT metabolites. The fragmentation of this derivative under EI conditions is shown on figure 1d. Specificity was very good because of numerous diagnostic ions, but the direct consequence was the lowest sensitivity compared to the di-TMS derivative.

Purification

At first, an analytical approach based on a C18 solid phase extraction (SPE) and a liquid-liquid extraction (LLE) was developed. However, it was obvious that this approach can be acceptable only if the analyte concentration overload the ppb level or if a very specific technique as MS/MS or high resolution measurement was used. The goal being to reach detection limits below 100 ppt level and to analyse a large number of samples with an acceptable cost, the use of more sophisticated purification technique as well as more common

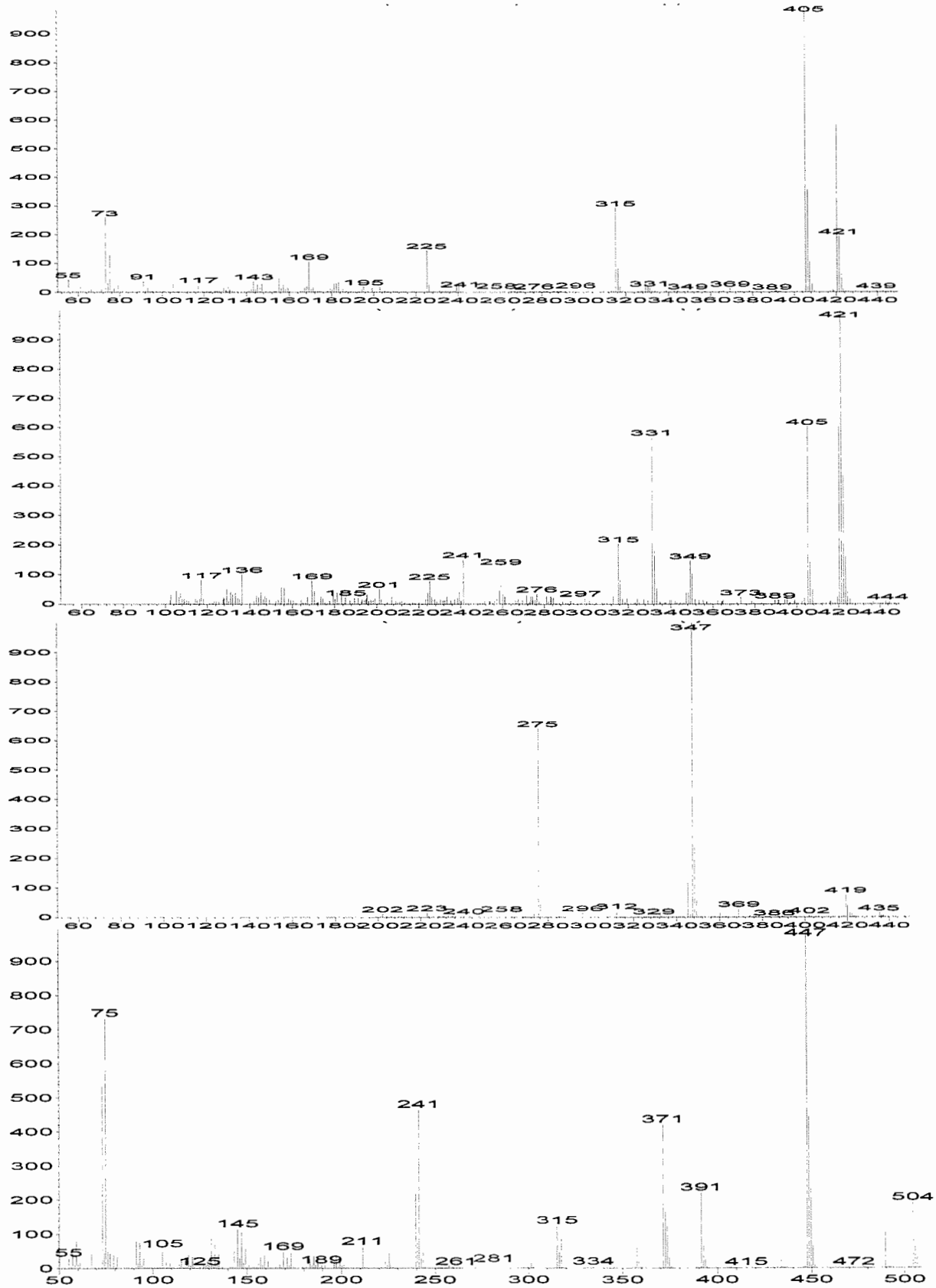

Figure 1 Mass spectra of derivatised 19-norandrosterone recorded in different ionisation modes. Up to down: TMS-EI, B: TMS-PCI (NH_3), C: TMS-NCI (NH_3), D: TBDMS-EI.

MS detector, i.e. quadrupolar mass filter, led us to change our analytical strategy. In a first time a silica SPE column was added to the procedure as final step. The results were correct with single quadrupole mass spectrometer but became insufficient in some urines at levels

near 0.1 ppb (figure 2a). Efficient diagnostic was guaranteed at this level only when a semi-preparative HPLC column was introduced; the chromatography was based on an aminopropyle stationary phase. The clean-up was impressive (figure 2b) so that almost no interferences disturbed interpretation of the ion chromatogram. Purification was so deep that the two metabolites can be separated one from the other. In these conditions the detection limit was estimated at 20 pg/ml on the m/z 405 fragment ion, S/N >3.

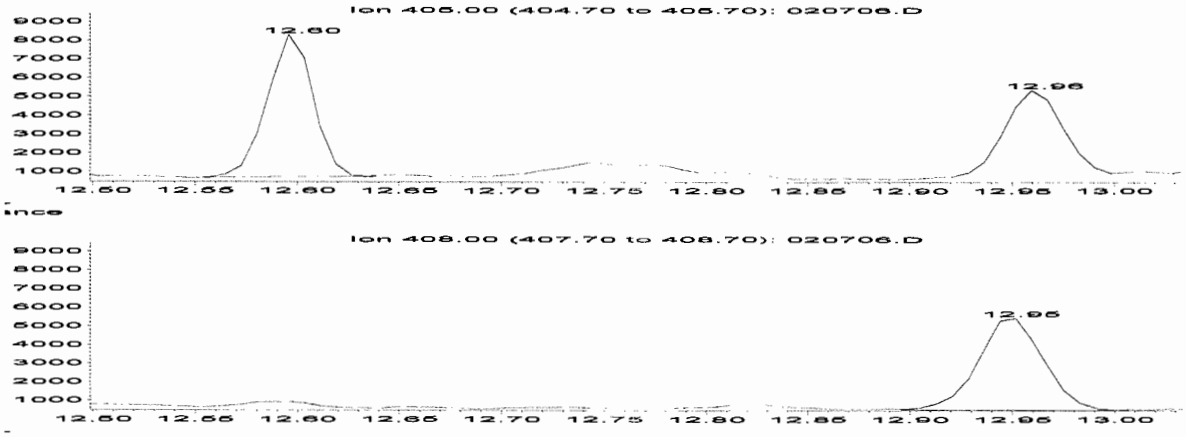

Figure 2 19-NA and 19-NE ion chromatograms of 0.1 ppb spiked urine purified thanks to semi-preparative HPLC. m/z 405 (up): 19-NA (tr=12.60 min) and 19-NE (tr=12.96); m/z 408 (down): 19-NE-d_3 (tr=12.95).

Deconjugation step

Several enzyme preparations were checked on real urine samples taken from adult males who has received oral administration of nandrolone. The best results were obtained with the β-glucuronidase from *E. coli* and the arylsulphatase/β-glucuronidase mixture from *Helix pomatia*. The deconjugation yield was better and less interferences were observed with the β-glucuronidase from *E. coli* (figure 3).

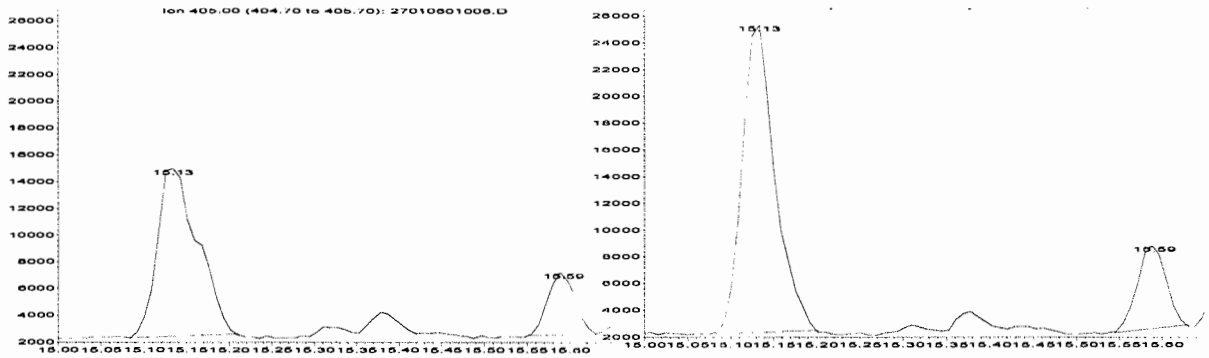

Figure 3 19-NA/19-NE incurred urine sample. Comparison of m/z 405 ion chromatogram profiles depending on enzymatic deconjugation procedure (left: *Helix pomatia*; right: β-glucuronidase from E. *coli*; 19-NA: 15.13 min; 19-NE:15.59 min).

Detection of 19-norandrosterone

Urines of volunteer adult males working in the laboratory were analysed according to the analytical procedure described before. On figure 4, two normal profiles were shown, one corresponding to an individual urine who did not excrete at this moment 19-norandrosterone nor 19-noretiocholanolone (ion chromatogram A), the other who excreted around 0.3 ppb of 19-norandrosterone (ion chromatogram B). A signal was detected for 19-noretiocholanolone but the signal to noise ratio was low.

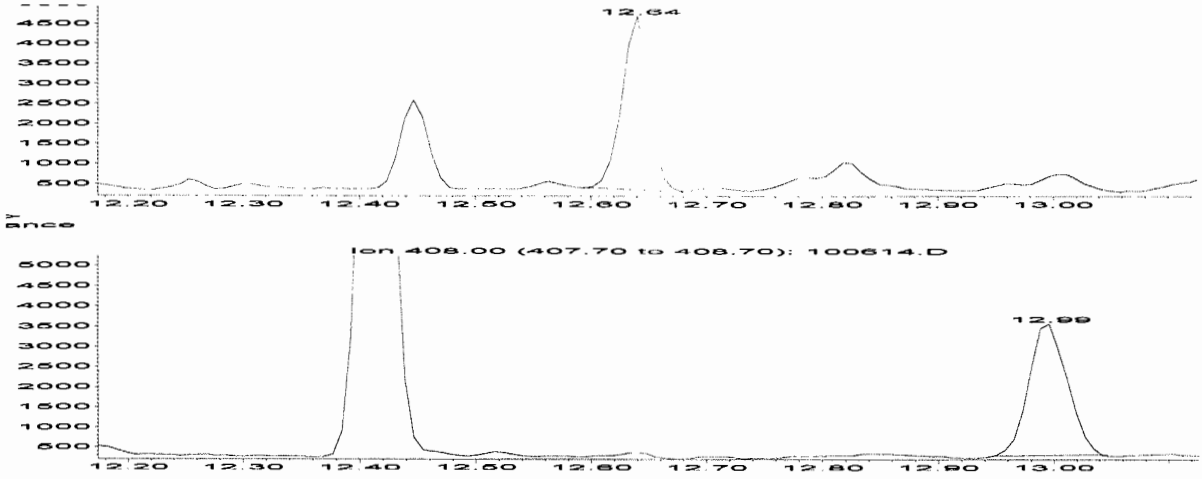

Figure 4 Detection of endogenous 19-norandrosterone in adult male urine. Ion chromatograms (up: m/z 405 for 19-NA (12.64 min) and 19-NE (13.00 min; down: m/z 408 corresponding to 19-NE-d_3 (12.99 min)).

Identification of 19-norandrosterone

The signal observed on the base peak chromatogram has to be confirmed according to the basic analytical rules (IOC or 93/256/EEC council directive [32]) to be considered unambiguously as corresponding to 19-norandrosterone.

Co-chromatography

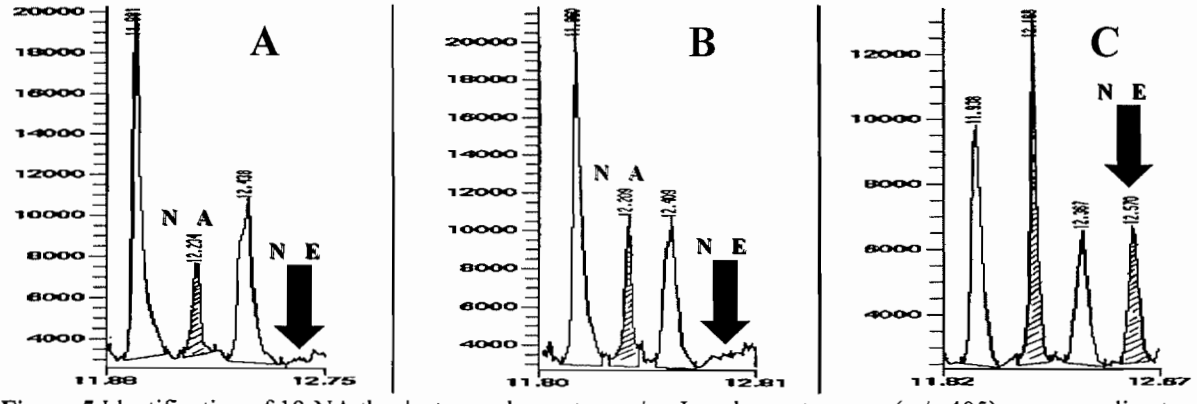

Figure 5 Identification of 19-NA thanks to co-chromatography. Ion chromatograms (m/z 405) corresponding to a healthy volunteer urine (A), spiked with 1 ng (B) and 5 ng of TMS-derivatised 19-NA and 19-NE (C).

Because of the very low concentration of these metabolites in urine, classical approaches were difficult to apply. The first procedure followed was a rapid and simple one: co-chromatography. Increasing concentrations of TMS-derivatised 19-NA and 19-NE standards were added to the previous derivatised extract and successively injected. The resulting mass chromatograms are shown on figure 5. Increasing of the 19-NA signal without any splitting in two, Gaussian peak symetry were in favour of 19-NA identification.

Ion ratios and parallel derivatisation

The diagnostic ion chromatograms m/z 420, 405 and 315 (A and B) and the SIM mass spectrum (C and D) of a 19-NA spiked sample (up) and of an analysed urine (down) are shown on figure 6. Target ion ratios (± 10% tolerance in EI) and relative retention times (± 0.5%) were fulfilled (according to the 93/256/EC directive). The same approach was applied on the TBDMS derivatised extract: the acquired informations permitted to verify once again the criteria fulfilment of the new retention time and diagnostic ions.

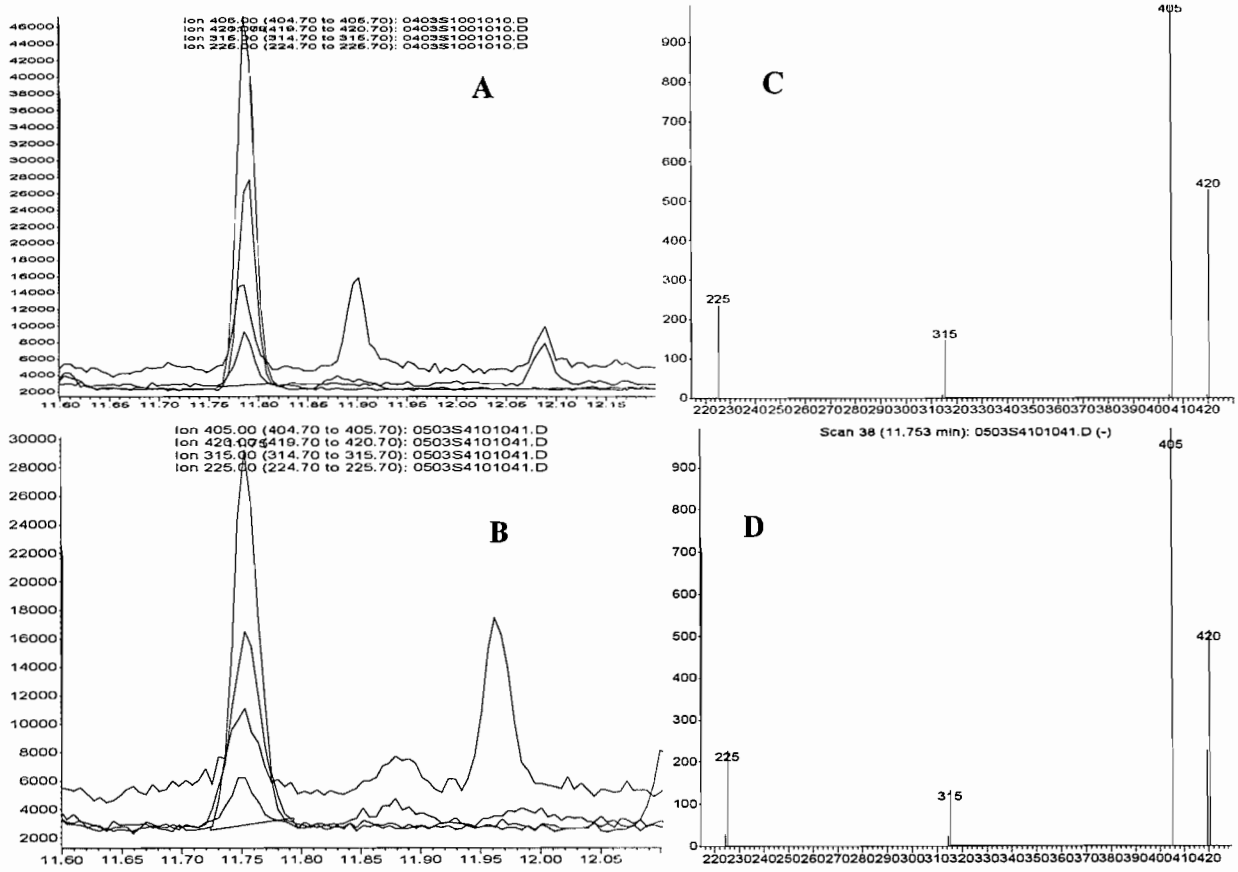

Figure 6 Identification of 19-NA according to 93/256/EEC directive criteria. (A: ion chromatograms of a spiked urine. C: corresponding SIM MS. B: ion chromatograms of a suspicious urine. D: corresponding SIM MS).

High resolution measurement confirmation

Complementary analysis was achieved on double focusing mass spectrometer permitting high-resolution measurements (R=10.000 at 10% valley definition). Mass chromatograms corresponding to a blank and to a suspicious urine are respectively shown on figures 7A and 7B. Exact masses of 4 diagnostic ions were recorded for the two metabolites (420.2880, 405.2645, 315.2144 and 225.1643). The four ions of 19-NA in the good ratios were observed at the expected retention time.

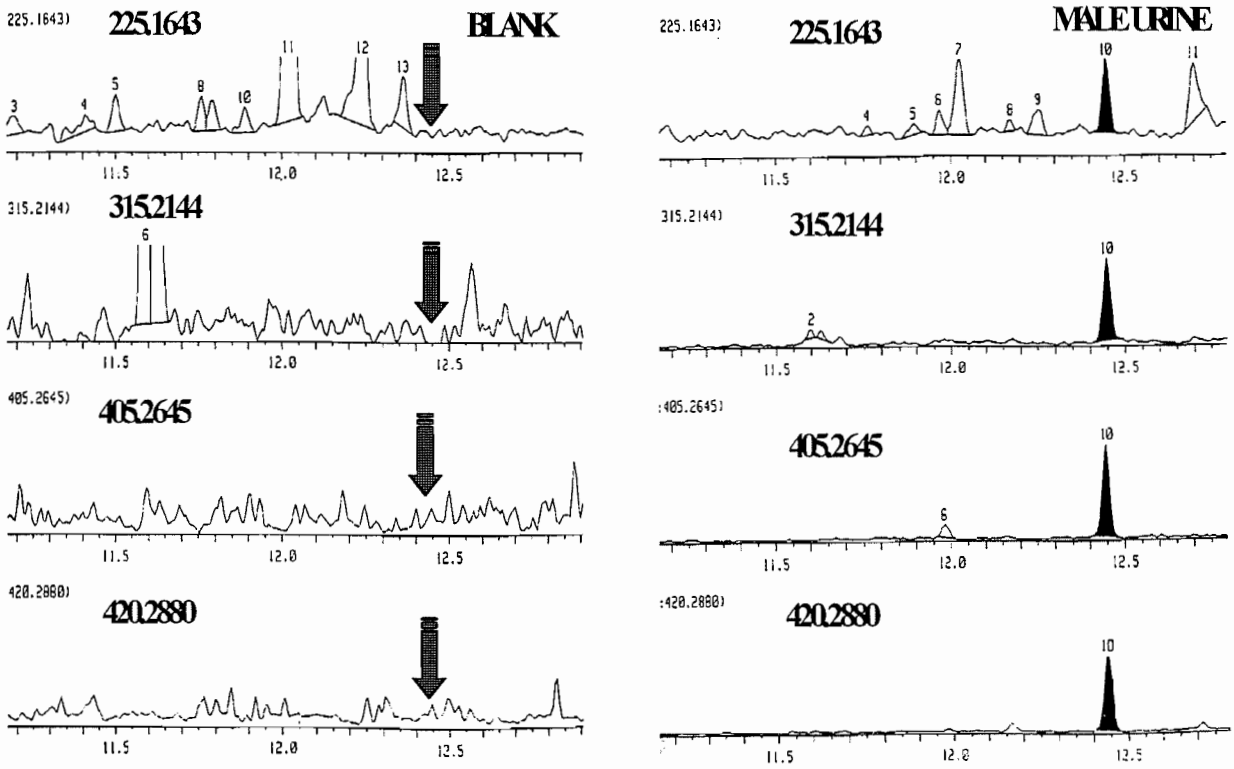

Figure 7 Identification of 19-NA thanks to high resolution mass spectrometry measurement. Ion chromatograms (from up to down m/z 225.1643, 315.2144, 405.2645 and 420.2880) corresponding to a blank (left) and to a suspicious urine (right).

Quantification of 19-norandrosterone

Urines of eight volunteer adult males working in the laboratory were taken at different moment of the day (7h, 11h, 17h and 23h). Eighteen on 32 urines (more than 50%) were proved to contain 19-NA (Tab.1); no 19-NE was observed above the detection limit (LOD) of the method (0.02 ng/ml). The calibration curve reported on the y-axis the m/z 405 signal and on the x-axis the concentrations of the four extracted spiked urines (0, 0.1, 0.3 and 0.5 ng/ml). In the studied population, the measured concentrations varied between the detection limit, i.e.

0.02 and 0.6 ng/ml (Table 1). No correlation could be done between the concentrations and the sampling hour or the age of the volunteers; excretion of 19-NA was almost observed all the time in the first urine of the day. This 1-day monitoring on 8 non-sportive individuals was extended to 1 top-level athletes on 3 months.

Table 1

Suspected 19-NA in 32 urine samples from healthy male volunteers (+: concentrations between LOD and 0.2 ng/ml, ++: concentrations between 0.2 ng/ml and 0.6 ng/ml).

AGE (years)	7 h	11 h	17 h	23 h
19	+	< 0.02	< 0.02	< 0.02
20	+	++	++	++
21	+	< 0.02	< 0.02	< 0.02
24	++	+	+	++
30	+	< 0.02	++	< 0.02
46	++	+	+	< 0.02
51	< 0.02	< 0.02	+	+
52	< 0.02	< 0.02	+	< 0.02

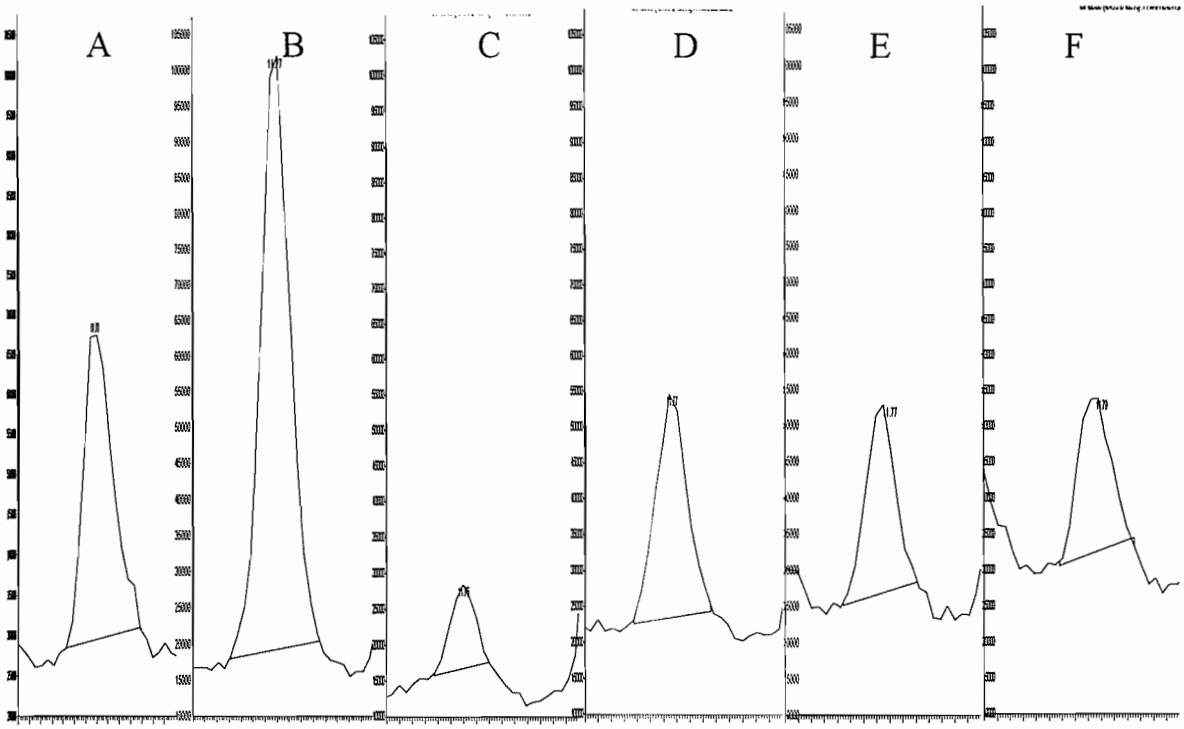

Figure 8 Quantification of 19-norandrosterone in one male person. M/z 405 ion chromatograms corresponding to the urine sample of a sportsman sampled during 2 months. From the left to the right: A=12 January/0.15ppb,

B=19 February/0.47 ppb, C=25 February/0.06 ppb, D=4 March/0.12 ppb, E=9 March/0.13 ppb, 18 March/0.11 ppb.

The m/z 405 ion chromatogram signals and the corresponding determined concentrations are shown on figure 8. The values varied between 0.06 and 0.47 ng/ml, with a mean value of 0.17 ng/ml.

Conclusion

A specific and sensitive analytical method has been developed in order to detect and to identify low concentrations of 19-NA and 19-NE. 19-NA was detected and identified in 18 urine samples on a population of 32 individuals guaranteed non treated with nandrolone. The concentrations found in the eighteen 19-norandrosterone detected samples did not exceed 0.6 ng/ml with main values belonging to the 0.1-0.3 ng/ml interval. The presence of 19-NT metabolites in human urine must be elucidated. Some hypothesis on the possible origin of 19-NA and 19-NE should be verified as the influence of nandrolone contaminated meat consumption (pregnant cow, entire male pig, anabolised cattle), as well as the consequences of an intense prolonged effort and competition stress. However that may be, it appears necessary now to determine natural levels on a large population as well as to elucidate endogenous synthesis pathway, and in any case to determine new criteria based either on quantitative measurement or ratio between phase I and/or phase II metabolites.

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