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19-Norandrosterone Origin: A Simple Procedure for GC/C/IRMS Sample Preparation by HPLC

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Introduction

The use of anabolic androgenic steroids (AAS) is prohibited in sports and these compounds are included in the Prohibited List of forbidden compounds and methods published and periodically updated by the World Anti-Doping Agency (WADA) [1]. The AAS included in the list can be divided in three main groups: those with a synthetic chemical structure derived from testosterone (i.e. methyltestosterone or stanozolol), the "pseudo-endogenous" (or "endogenous-like") compounds, with an identical chemical structure compared to the corresponding endogenous compounds (i.e. testosterone itself or androstenedione), and finally those compounds that, even if being originally known as synthetic, may be present in some very specific circumstances in the urine samples of the athletes (i.e. 19-norandrosterone, boldenone or formestane). For the detection of AAS routine methods based on gas or liquid chromatography coupled to mass spectrometry (MS) [2-4] have been established while for reporting a result as an adverse analytical finding (AAF) for the last two groups of compounds the confirmation of their origin by isotope ratio mass spectrometry (IRMS) has become mandatory[5].

19-norandosterone (19-NA) is in humans the main metabolite of synthetic 19-nortestosterone (nandrolone), 19-norandostenedione and 19-norandostenediol [6-10], but it can be also produced as a minor metabolite of norethandrolone or ethylestrenol [11] or by the administration of some progestagenic drugs as norethisterone [12]. In addition to the previously mentioned synthetic origin, the naturally occurring production of 19-NA has been demonstrated in animals and human males [13-18]. In females, due to the production of large amounts of estrogens during pregnancy or ovulation, 19-NA can be produced as a by-product of aromatization [19, 20] leading to detectable concentrations of 19-NA in urine. In males this metabolic route is less expressed leading to the production of trace amounts of 19-NA only [21]. The origin of 19-NA in samples showing concentrations between 2 and 10 ng/mL must be confirmed by IRMS[22]. In some very rare and particular conditions the *in situ* formation

of 19-NA (by endogenous steroids 19-demethylation) in urine specimens after a long term storage of the samples has been described [23]. Finally it has been reported, even if extremely improbable, that the consumption of meat (i.e from non-castrated pigs or boars) can result in the later excretion of nandrolone metabolites into urine since 19-NA is endogenous in some species [15, 24].

Experimental.

Standards and reagents

The standards of testosterone (17β-hydroxy-4-androsten-3-one, **T**), androsterone (3α-hydroxy-5α-androstan-17-one, **A**), 19-norandrosterone (3α-hydroxy-5α-estrane-17-one, **19-NA**) were purchased from NMIA (Pymble, Australia), methyltestosterone (17α-methyl-4-androsten-17β-ol-3-one, **MT**), estradiol (estr-1,3,5(10)-triene-3,17β-diol, **E2**) and estrone (estr-1,3,5(10)-triene-3-ol-17-one ; **E1**) from Sigma-Aldrich (Milano, Italy) , and androstenedione (4-androsten-3,17-dione, **AED**) and 5α-androstan-3β-ol from Steraloids (Newport, RI, USA).

All reagents and solvents (sodium bicarbonate, potassium carbonate, sodium phosphate, sodium hydrogen phosphate, *tert*-butylmethylether (TBME), acetonitrile, methanol, n-pentane, cyclohexane and isopropanol) were of analytical or HPLC grade and provided by Carlo Erba (Milano, Italy). β-glucuronidase from *Escherichia coli* K12 was from Roche Diagnostic (Mannheim, Germany). Water was from a Milli Q water purification system (Millipore S.p.A, Milano, Italy).

Cytochrome CYP19 (aromatase) enzyme expressed form c-DNA (BD-Supersomes™) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) regenerating system were from BD Biosciences (Buccinasco, Italy).

CO_2 reference gas (Solgas, Monza, Italy) for isotope ratio mass spectrometer calibration was calibrated against underivatized steroids (CU/USADA34-1) with certified delta values traceable to VPDB, obtained from Prof Brenna (Cornell University Ithaca, NY)[25].

CYP19 Metabolism incubations

For the metabolic studies, CYP19 preparation containing 20 mg/mL of protein in 0.1 M phosphate buffer (pH 7.4) were used. For the evaluation of the metabolic reaction, a reaction mixture containing 200 µL 0.5 M phosphate buffer (pH 7.4), 15 µL NADPH regenerating system, and 10 µL of substrate solution (testosterone or androstenedione at a final concentration of 10 µM) was incubated for 5 minutes at 37°C. The reaction was initiated with

the addition of 20 µL CYP19+reductase (to a final concentration of 20 pM). The samples were briefly mixed and then incubated at 37°C in shaking water bath. Aliquots of 100 µL were collected at different time intervals for the kinetic study (by GC/MS) and the whole incubation volume of additional incubations (1 mL) at 6 hours for the GC/C/IRMS analyses. After collection of each sample, the reaction was stopped with the addition of 100 µL acetonitrile and the samples were centrifuged for 5 minutes at 12,000 x g at 4°C.
Liquid/liquid extraction was carried out with TBME (5 mL), the organic layer taken to dryness and the dry residue reconstituted in 50 µL of a mixture water/methanol (50/50) containing methyltestosterone (100 µg/mL) used to check the repeatability of the elution times in the subsequent HPLC purification of the extracts.

Urine samples preparation

As previously described [26] due to the low amounts to be detected (below 3 ng/mL) up to 21 mL (3 x 7 mL) of urine may be needed for 19-norandrogens analysis by IRMS.
Urine samples were extracted with 10 mL of TBME after the addition of 1 mL of phosphate buffer (0.8M, pH 7) to separate the free from the conjugated fraction. Once the free fraction discarded, the hydrolysis was initiated by the addition of 50 µL of β-glucuronidase from *E. coli*. The hydrolysis was performed at 55 ºC during 60 min. After cooling, pH was adjusted to 9-10 with carbonate buffer (20%) and extraction performed with 10 mL of TBME. Once the solvent separated and taken to dryness, the final residue was dissolved in 50 µL of a mixture water/methanol (50/50) containing methyltestosterone (100 µg/mL) for subsequent HPLC purification of the extract.

HPLC sample purification

Purification for 19-norandrogens analysis .

Sample purification was performed using an Ascentis phenyl column from Supelco (Sigma-Aldrich, Milano, Italy) (15 cm, 4.6 mm, 3 µm) at 60 °C. Separation was programmed with a mobile phase composed with water (solvent A) and acetonitrile (solvent B). For compounds separation, an isocratic program was set up at 50 % B for 8 min then increasing to 100 % B in 0.01 min. The column was flushed for 7 min at 100 % B and finally re-equilibrated at 50 % B for 5 min for a total run time of 20 min. The flow rate was set at 1 mL/min. See Figure 1 for the collected fractions.

All separations are performed in an Agilent 1100 Series liquid chromatograph (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) and the selected fractions collected in a Agilent 1100 fraction collector. Separation conditions were established by monitoring the signal of a UV lamp at 192 nm (Agilent 1100 UV DAD detector). Fractions before and after the fractions containing the substances of interest were collected and analyzed in order to verify that all the peaks of interest were completely collected, otherwise an isotopic fractionation may occur and false $\delta\ ^{13}C$ values could be obtained in the GC/C/IRMS (see Figures 1 and 2).

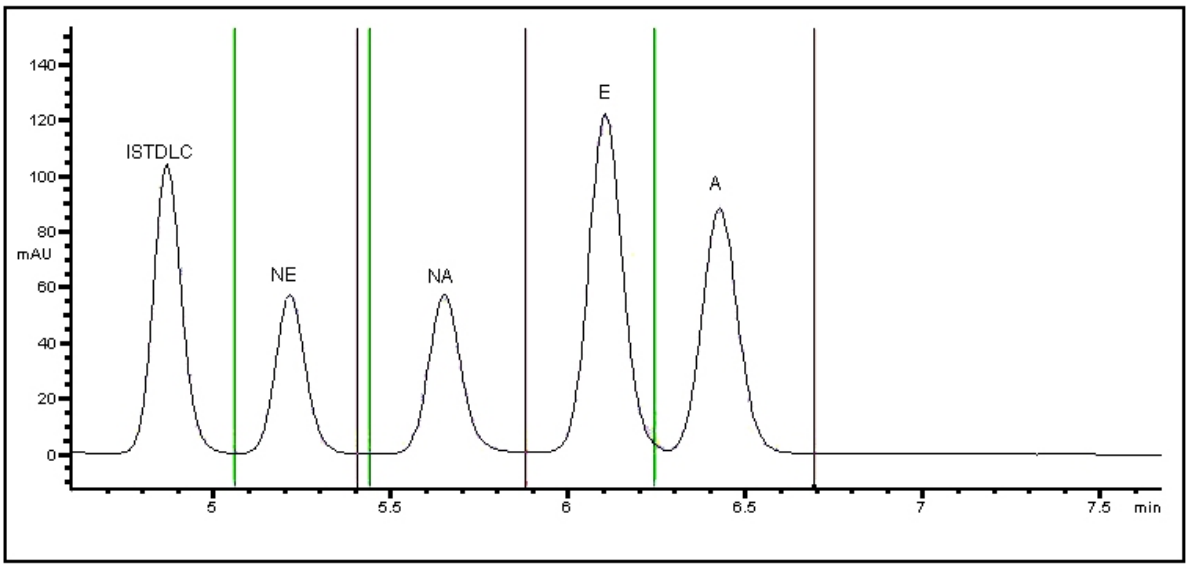

Figure 1. HPLC chromatograms for 19-norandrogens confirmation purification

Incubations with CYP19 were also purified by HPLC using a Discovery C18 column from Supelco (Sigma-Aldrich, Milano, Italy) (25 cm, 4.6 mm, 5 µm) at 38 °C. Separation was programmed with a mobile phase composed with water (solvent A) and acetonitrile (solvent B). For compounds separation, an isocratic program was set up at 38 % B for 26 min then increasing to 55 % B in 0.01 min, then to 65 % B in 4 min and kept at 65 % B for additional 4 min. The column was flushed for 6 min at 100 % B and finally re-equilibrated at 38 % B for 5 min for a total run time of 45 min. The flow rate was set at 1 mL/min..

After the HPLC procedure for sample purification was applied, the collected fractions were taken to dryness under a nitrogen stream. Before their analysis by GC/C/IRMS or GC/MS, the fractions were dissolved with an adequate volume of a mixture cyclohexane/isopropanol (4/1) containing 5α-androstan-3β-ol (20 µg/mL), according to the estimated concentration in the original sample, in order to get adequate signal for the IRMS analysis.

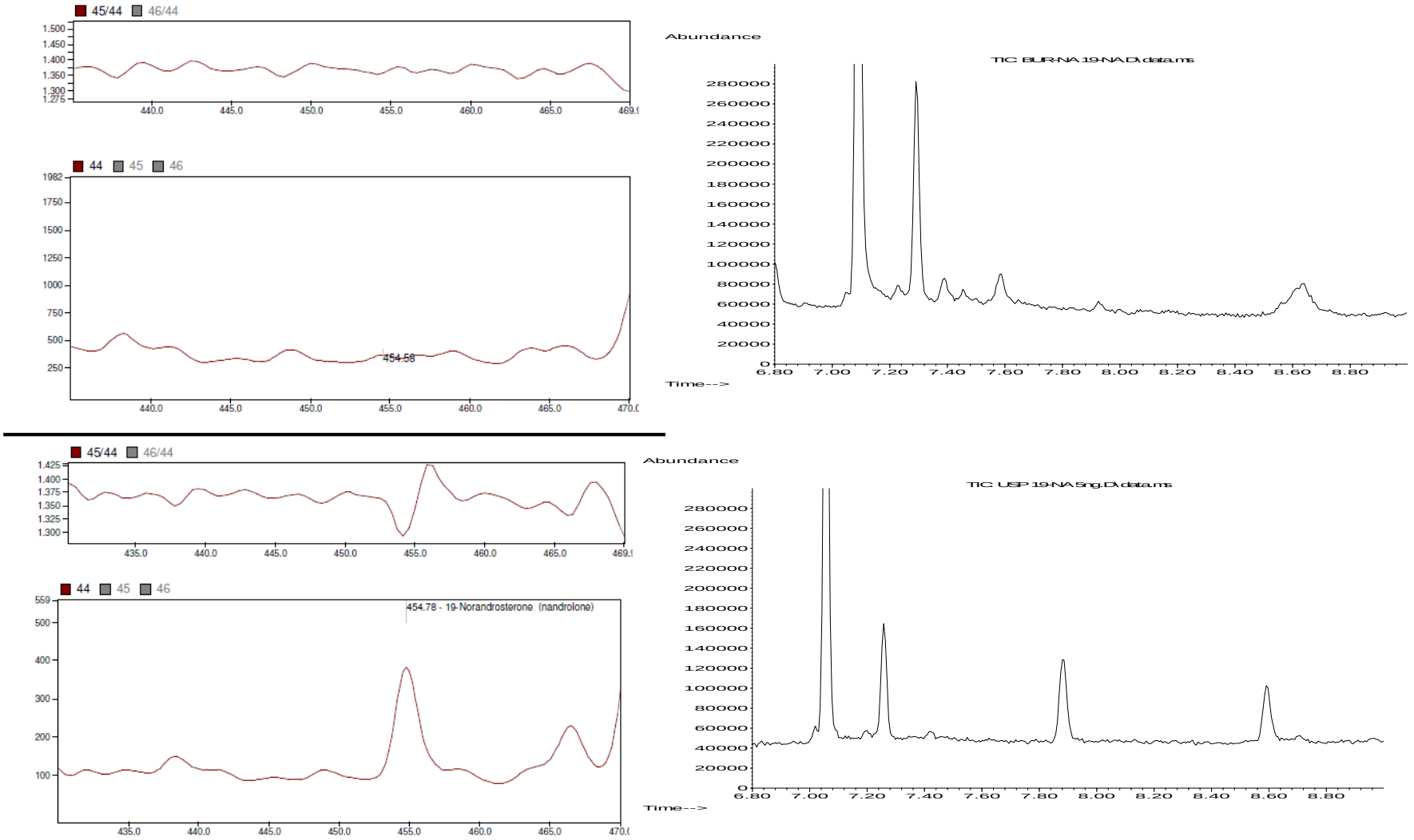

Figure 2. GC/C/IRMS (left) and GC/MS (right) analyses of a blank urine (up) and of a urine spiked at 5 ng/mL(down) after the sample purification process. The presence of 19-NA is indicated by the arrows and its elution time by dotted lines in the urine blank.

Instrumental analysis

GC/C/IRMS conditions

GC/C/IRMS analyses were performed in a HP6890 gas chromatograph (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) connected to a combustion furnace linked to a Thermo Delta V Advantage isotope ratio mass spectrometer (ThermoElectron, Bremen, Germany).
The chromatography was performed with a HP5MS (J&W Scientific) 5% phenylmethyl fused-silica capillary column (30 m x 0.25 mm i.d., 0.25µm film thickness). Compounds were separated using the following GC oven program: initial temperature 150 °C for 1 min, increased at 25 °C/min to 260 °C maintained 4 min, increased at 40 °C/min to 310 °C and kept 2.7 min at the final temperature. Helium was used as carrier gas at 2.1 mL/min and injection, 2 µL of extract, was performed in splitless mode at 280 °C.
The Thermo GC Combustion III interface (ThermoElectron, Bremen, Germany) was used with an oxidation furnace at 960°C. Generated CO_2 was dried on a Nafion^{TM} membrane before entering the mass spectrometer. High purity oxygen gas was flushed trough the furnace for 3 s prior to an analysis sequence. The calibration of the reference gas was performed by the analysis of certified reference material (see section 1.1).

GC/MS instrumental conditions

The purity of the extracts analyzed by GC/C/IRMS was verified by their parallel analysis in a HP6890 gas chromatograph coupled to HP5973 mass spectrometer (Agilent Technologies SpA, Cernusco sul Naviglio, MI, Italy) equipped with the same type of column and under the same chromatographic conditions used for IRMS analysis. The chromatographic profile was equivalent to the one obtained on the isotopic analysis except the retention time lag due to dead volumes on the GC/C/IRMS system. The extracts (1 to 2 µL) were injected in splitless mode and the analyses were performed in full scan (range m/z 40-550).

Results.

The HPLC purification developed permitted to obtain extracts of adequate purity for GC/C/IRMS analysis. Figures 1 and 2 show the HPLC separation of the compounds of interest and the analysis of a blank urine and the same urine spiked with 5 ng/mL of 19-NA, analysed after HPLC purification by GC/CIRMS. The purity of the extracts was verified by GC/MS. See [26] for overall validation details. The method was applied to urine samples collected *in vivo* during a pregnancy using androsterone as endogenous reference compounds (ERC). A systematic delta-delta difference of 2.5 units was observed. Regarding the *in vitro*

experiments, after the incubation of 10 mM of AED with the aromatase system an increase of the formation of T, E1 and E2 was observed with the parallel decrease of the substrate. Maximum response of the products formed was achieved at 6h. The formation of T and E2 are a consequence of the presence of the reductase present in the enzymatic system (see figure 3). Similarly, the incubation of 10 mM of T gave the formation of E1 with a maximum response at 6h and E2 at 18h and to a lower extend of AED. (see figure 3). Incubations were repeated at least in triplicate (single incubation time; 6h) in order o get enough material to be analyzed by GC/C/IRMS. As shown in table 1 after the incubation of the substrates, the systematic formation of products with lowered $\delta^{13}C$ values was observed leading to $\Delta\delta$ values that are for some of the pairs of compounds in agreement with the *in vivo* observations and would sustained that a fractionation during the enzymatic reaction occurred.

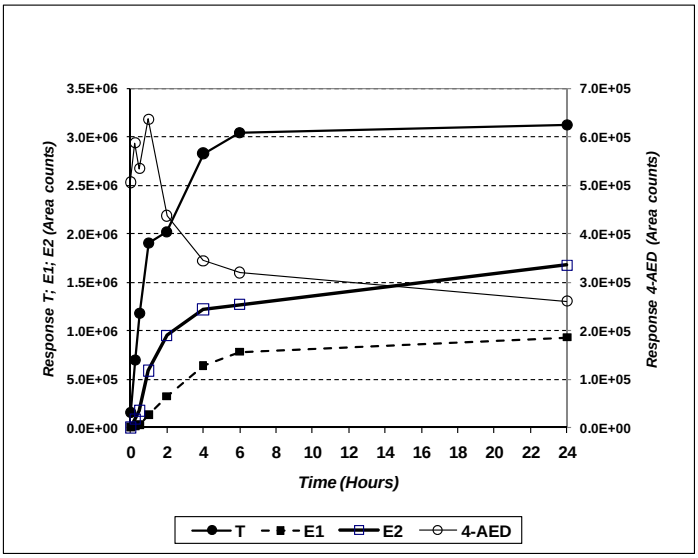

Figure 3. 10 µM Incubation of androstenedione (4-AED) with human CYP19 + P450 reductase Supersomes ™ (BD Biosciences)

Table 1. AED incubation (6h) with human CYP19 + P450 reductase Supersomes ™ and changes on the $\delta^{13}C$ values of the products formed

$\delta^{13}C$‰	Mean	SD	n
T	-32.4	0.40	6
E2	-32.0	0.29	5
4-AED	-29.2	0.25	6
E1	-29.0	0.63	6
Δδ	**Mean**	**SD**	**n**
AED-E2	2.7	0.21	5
AED-E1	-0.2	0.46	6
AED-T	3.2	0.29	6

Discussion.

The confirmation of the exogenous origin of 19-NA in human urine is mandatory for samples showing concentrations between 2 and 10 ng/mL before reporting an adverse analytical finding[22]. Sample preparation methods able to reach the adequate extract purity and sensitivity permitting a GC/C/IRMS analysis are available [26]. In anti-doping analysis, several endogenous reference compounds (ERC) have been proposed by different authors in order to have a reference compound with a non-modified delta value to compare with the analyte of interest and produced by the same individual. This strategy has been included in the WADA regulations and a delta-delta ($\Delta\delta$) value higher than three (^{13}C δ analyte – ^{13}C δ ERC > 3) has been proposed to disclose the endogenous origin form the synthetic one[5]. In the specific case of 19-NA, androsterone (A) has been suggested as the most appropriate ERC [27] since it is present in urine in large amounts, its detection is performed routinely for confirmatory IRMS analysis of synthetic androgens and because it has been postulated as the origin of 19-NA in the so-called active urines [23].

A systematic and constant difference of aproximatively 2.5 delta units in the delta-delta values ($\delta^{13}C_{19\text{-}NA}$ - $\delta^{13}C_A$) was observed in parallel with the formation of estrogens during pregnancy [26]. This observation has also been made by others in samples where 19-NA of endogenous origin was detected, in most cases linked to aromatization [18].

Metabolic fractionation in endogenous steroids production has been described due to the endogenous synthesis itself. Physiologically, different $\delta^{13}C$ values have to be assumed for different compounds. [28]. This involves Phase I but also Phase II reactions [29]. There is a fractionation effect during the C4-5 double bound reduction producing 5β-reduced metabolites with more depleted δ values. [28, 30-32]. Differences associated to the gender have been described. The $\delta^{13}C$ values of the 5β-compounds (Etiocholanolone (Et), 11OHE, P2) are nearly identical for males and females. In contrast, the 5α-steroids A and 11OHA exhibits a ^{13}C depletion in females. Furthermore, Et is generally characterized by lower $\delta^{13}C$ values in both sexes [28, 32].

For most if not all the examples reported before, the fractionation was observed over compounds that differ in the degree of oxidation of the molecule but were constant in term of number of C atoms.

Fractionation on the metabolism can be relevant for the selection of the adequate ERC to be used to calculate the $\Delta\delta$ values [33] and on the establishment of the reference and cut-off values.

In order to investigate the observed fractionation during the formation of 19-NA (18 C atoms) from androgens (19 C atoms) and its relevance in anti-doping analyses, *in vitro* experiments using recombinantly expressed c-DNA enzymes (BD-Supersomes^{TM}) have been performed using testosterone and androstenedione as substrates (see figure 3). The expressed enzymes consisted in cytochrome P450arom (CYP19) and NADPH-cytochrome P450 reductase, both necessary for the enzymatic activity of the system. Both aromatase and reductase activities are present simultaneously *in vivo* as in the *in vitro* enzymatic model applied. This system can be used as an alternative to the placental microsomal aromatase assay recommended as the initial *in vitro* aromatase inhibition screening assay and has been validated by the United States Environmental Protection Agency (U.S. EPA) on the frame of implementing an Endocrine Disruptor Screening Program (EDSP) [34]. The results obtained supports the hypothesis that endogenous 19-NA is produced as a by-product of the synthesis of estrogens.

It would be interesting to verify whether in the so-called active urines [23], where 19-NA has been produced, the delta changes observed during the aromatization process also occur. This would probably help to better understand the mechanism involved in the 19-demethylation of androgens for the formation of 19-NA in the active urines and to verify if the mechanisms would be different from the postulated one.

Conclusions.

Some endogenous steroids metabolic fractionation of the delta values has been described. This can be relevant when establishing the delta-delta criteria to discriminate the synthetic from the endogenous origin of a given endogenous steroid found in urine samples.

The formation of trace amounts of 19-NA as a by-product of the aromatization process of androgens has been detected *in vivo* and a consistently high delta-delta value been observed. This initial observation has now been confirmed by *in vitro* experiments where products with a depleted delta value have been obtained after the incubation of androgens with a purified CYP19 enzyme systems. These observations should be taken into account when evaluating the IRMS results obtained during a 19-NA confirmation and should be considered in the international antidoping regulations.

Acknowledgments.

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