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In-situ steroids 19-demethylation *vs* aromatization

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Abstract

The presence of 19-norandrosterone in urine samples may have several origins and under specific circumstances, additional testing and reporting may be required as the detection of pregnancy (hCG test) or progestogens consumption in females. In all circumstances the use of isotope ratio mass spectrometry (GC/C/IRMS) should permit to differentiate the endogenous from a synthetic origin of the 19-NA detected. With the improvement of the samples transport to the Laboratories and the reduced time for the release of the results (10 working days), the detection of active urines is more and more infrequent.
We describe a case of a recent urine sample showing the "usual" characteristics for an active urine [turbidity , 19-NA/19-NE (1.2) < A/Et (1.5) and high specific gravity (1.027)] where the IRMS analysis was conducted. The sample showed 19-NA compatible with an endogenous origin ($\Delta\delta_{19NA\text{-}A}$< 3‰; ca. 0.1‰) and the stability test demonstrate that the sample was unstable (formation of deuterated-19-NA), explaining the 19-NA finding. The Δδ values observed in our laboratory and by others during pregnancy are between 2 and 3 and *in vitro* experiments with CYP19 incubations of androgens seem to confirm that an isotope discrimination occurs during aromatization. The data of the case presented, and previous data already published, suggest that the in-situ 19-demethylation of A to 19-NA in urine is produced by a 19-demethylase different from the aromatase, originated by the growth microorganisms in the urine sample

Introduction

Nandrolone and its precursors detection is based on the identification of the main urinary metabolite, 19-norandrosterone (19-NA) in a concentration greater than the Decision Limit (DL), as established in the World Antidoping Agency (WADA) DL Technical Document [1]. The presence of 19-NA in urine samples can be originated by the external administration of nandrolone or nandrolone precursor, of progestogens (i.e. norethisterone) or wild boar/non-castrated pigs meat consumption. There is an endogenous productions under particular physiological conditions (i.e. ovulation, pregnancy). Finally the ex-vivo production in urine by in-situ by 19-demethylation of androsterone (A) (active urines).
In all circumstances the use of isotope ratio mass spectrometry (GC/C/IRMS) should permit to differentiate the endogenous from a synthetic origin of the 19-NA detected.

Experimental

GC/MS analysis
Two mL of sample were hydrolyzed with 30 µL of enzyme (*E. coli*) for 1h at 55°C after the addition of the internal standard (mixture of deuterated standards) and 750 µL of phosphate buffer (0.8 M). The free and deconjugated steroids were then extracted with 5 mL of *tert*-butylmethyl ether at pH 9-10 after 250 µL of $Na_2CO_3/NaHCO_3$ (20%) was added. The organic solvent was taken to dryness and the final residue dissolved in 50 µL of MSTFA/NH_4I/2-mercaptoethanol (1000:2:6). Two µL were injected in a GC/MSD in SIM acquisition mode for the screening analysis.

"Active urine" stability test
Two urine aliquots of 500 µL were added with 5 µg of androsterone-D4 and etiocholanolone-D5 respectively. The samples were incubated at 37°C overnight. After incubation the samples were extracted with the same procedure applied for the quantification of 19-NA by GC/HRMS using 19-NA-D4 or 19-NE-D4 as ISTD.

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IRMS

GC/C/IRMS was applied as described in [2] starting from 21 mL of urine.

Microbiological investigation

Urine sample were inoculated on Sabouraud agar (SAB) for fungi or Trypticase soy agar (TSA) for bacteria and incubated at 37°C and 27°C for 24h or 1 week respectively. After 24h the Gram-positive colonies were selected by Gram staining, and inoculated in a selective and differential medium.

Results and Discussion

A sample from a male football player showed the presence of 19-NA (ca. 2.9 ng/mL, screening data) and a 19-NA/19-NE (0.7) < A/Et (1.5). High turbidity and a relatively high specific gravity (1.027), additional characteristics of "active urines", were also observed [3]. The time between sample reception and the adverse analytical detection was of 8 days. In the past, the "activity" was linked to long term storage of the samples but although the reporting time has been reduced these degradations cannot be excluded.

We decided to apply in parallel the procedure for quantification and active urine test as well as to apply for the GC/C/IRMS confirmation procedure.

Figure 1 shows the results of the activity test, confirming the *ex-vivo* capacity of the sample to generate 19-NA. Deuterated 19-NA and 19-NE were obtained after the incubation of deuterated androsterone and etiocholanolone respectively.

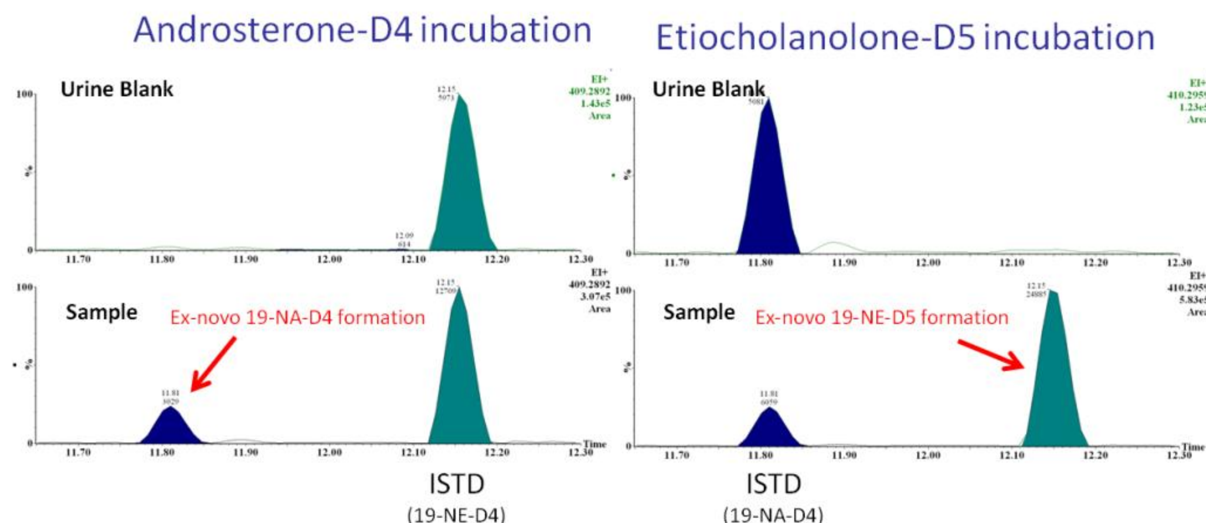

Figure 1. GC/HRMS active urine test showing the formation of 19-NA-D4 (using 19-NE-D4 as ISTD) and 19-NE-D5 (using 19-NA-D4 as ISTD , shown as the +1 isotope mass fragment) in each specific incubation with the suspicious sample and not appearing in the urine blank samples

The analysis by IRMS (see Table 1) demonstrated the non-synthetic origin of the 19-NA detected in the sample (established at 3.4±0.2 ng/mL after the quantitative confirmation). The observed Δδ values were far below 3. In previous observations during the formation of 19-NA during pregnancy, higher Δδ values have been described. The loss of the C19 methyl group during the aromatization process tend to produce higher Δδ values as some preliminary *in vitro* data with isolated CYP19 enzymes seems to support [4]. The presence of aromatases in the urine is difficult to explain while the enzymatic activity derived from microorganism growth is well known. We can imagine that the involvement of enzymes of different origin during the *in-vivo* or *ex-vivo* 19-NA formation may be the responsible of such observation.

The microbiological analysis of potential pathogens in the sample showed the presence of *Enterobacter sp.* Further studies with controlled incubations of selected microorganisms are in progress in order to elucidate the potential responsible(s) of the 19-demethylation and the isolation of the enzyme(s) involved.

	$\delta^{13}C$ (‰)	$\Delta\delta$ ERC-19-NA
19-NA	-21.9	
A	-22.0	-0.1
11-Keto	-23.0	-1.1
PT	-21.4	-0.5

Table 1. GC/C/IRMS data on the suspicious sample (19-NA: 19-norandrosterone, A: androsterone; 11-Keto: 11-keto-etiocholanolone; PT: pregnanetriol)

Conclusions

The results obtained by GC/C/IRMS demonstrated that the detected 19-NA was not of synthetic origin. The formation of deuterated 19-NA demonstrated that the original finding was due to an “active urine”.
The Δδ values observed in our laboratory and by others during pregnancy are between 2 and 3 and *in vitro* experiments with CYP19 incubations of androgens seemed to confirm that an isotope discrimination occurs during aromatization.
The data of the case study presented, and previous data already published, suggest that the in-situ 19-demethylation of A to 19-NA in urine is produced by a demethylase different from aromatase, originated by the growth a microorganism in the urine sample.

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