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The usefulness of GC/C/IRMS in determining the origin of low levels 19-NA – application in routine analysis.

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19-norandrosterone glucuronide (19-NA), main urinary metabolite of 19-nortestosterone, 19-norandrostenedione and 19-norandrostenediol, and minor metabolite of norethisterone[1], can also be produced, albeit in minute amounts, in males and females. Throughout pregnancy, 19-NA can be detected in urine samples at levels reaching approximately 15 ng/mL[2]. 19-nortestosterone is present in the intact boar; the consumption of meat-rich meal composed of non-castrated pig offal, although highly improbable, will lead to the excretion of 19-NA at levels exceeding the threshold[3]. Adverse analytical findings are reported when the concentration of 19-NA is greater than 2 ng/mL taking into account the uncertainty of the measure; the threshold, introduced in 1998[4], is adjusted to the specific gravity of the specimen.

Recently, the formation of 19-noretiocholanolone (19-NE) and 19-NA, the latter to a lesser extent, was reported in athlete's samples following incubation[5]. Urine samples in which 19-demethylation activity was noted, were often very concentrated and turbid. This crucial observation, confirmed by two different groups, prompted the revision of the criteria for reporting adverse findings to include verification steps in the few samples showing the criteria of unstable urines[6].

It is not possible to determine the endogenous or exogenous origin of urinary 19-norsteroids metabolites by the ratio of their 5α to 5β metabolites or by the ratio of the glucuro- and sulfoconjugates. The IRMS analysis may permit that differentiation. However, the reliable analysis of urinary 19-NA in trace amount by GC/C/IRMS was not possible without improving instrumental sensitivity and sample purification[7]. We wish to report here,

as a confirmation of the work already published by the Laboratory in Cologne[8], the results of the routinely applied GC/C/IRMS analysis of urine samples containing 19-NA in amount as low as 2 to 3 ng/mL in relatively low volume of urine sample. The IRMS analysis has been utilised to determine the endogenous origin of 19-NA present in athlete's samples as a result of 19-demethylation, thus confirming the occurrence of that reaction in athlete's urine samples.

Experimental

Purification of samples for GC/C/IRMS: The volume of urine sample is adjusted to the amount of metabolites present (5 to 15 mL). Solid phase extraction on Sep-Pak tC18 cartridges previously washed with methanol and water is made on 5 mL portions of urine; the steroids are eluted with methanol after rinsing with water and n-hexane. The hydrolysis is carried out in phosphate buffer 0.1M pH 6.9 with β-glucuronidase from *E. coli* type IX at 50°C for 1 hour. The extraction is carried out at pH 9 (carbonate buffer 1.0 M) twice with hexane. The aliquots are pooled, evaporated and dissolved in 25 µL of methanol. **Semi-Preparative reversed-phase HPLC:** the separation of the fractions containing the different steroids is carried out on a Agilent Zorbax SB-C_{18} column (5 µm, 9,4 X 250 mm) with UV detection at 192 and 245 nm; the initial composition of the solvent is Acetonitrile/Water 60% (v/v) raising to 65% in 30 min and to 95% in 1 min at a flow rate of 1,6 mL/min. The products recovered and the collection times are as follows: pregnantriol (17 – 18.3 min), 19-NA (20.8 to 22.2 min), etiocholanolone (22.2 to 24 min), androsterone (24.5 to 26 min) and pregnandiol (28 to 30 min). The solvent is evaporated to dryness, the residue is dissolved in 8 µL of TBME; underivatized purified extract are analysed as such. **GC/C/IRMS analysis:** The Finnigan Delta^{plus} XP (now available from Thermo Scientific) is equipped with Agilent 5973 GC/MSD on which a HP-5MS column (25 m x 0.20 mm, 0.33 µm film thickness) was installed. The reference gas is certified CO_2 (purity >99.9995%) Grade SFC, Air Products UN1013; verification of Delta values with alcanes "Mixture C" (C-17, C-19, C-21, C-23, C-25) obtained from Geol. Science (Indiana)[9]. Each sequence of injections includes (i) authentic standards of 19-NA, 19-NE (10 ng), androsterone, etiocholanolone, pregnantriol, pregnandiol (100 ng); (ii) our negative control urine sample consisting of a reference collection of "natural" 19-NA collected following ingestion of intact pig offal; (iii) steroids isolated from the urine sample; (iv) standards isolated by HPLC (to monitor possible

fractionation during isolation). The signal of 19-NA must be greater than 200 mV (rt 1341 ± 15 sec). The identity of the steroids analysed is checked by GC/MSD running on the same column with the same chromatographic conditions.

Results

Formation of 19-norsteroid metabolites in athlete's urines: Further to the report by Grosse et al. of 19-demethylation activity in some samples, we started submitting all samples received, prior to freezing, to the "activity" test proposed. In December 2004, we observed the clear formation of 19-NE and to a lesser extent of 19-NA in female athlete samples. The modifications of the profile (free and glucuroconjugated steroids) of aliquots incubated overnight are reported in Table 1.

Table 1: Modification of the excretion of 19-norsteroid metabolites following incubation at 37°C overnight of female athletes' urine samples collected in competition (held outside Canada)

		[19-NA]		*[19-NE]*	
		Free and Glucuroconjugated (ng/mL)			
Sample	Specific gravity	Initial	After	Initial	After
A	1,024	1,2	0,9	1,5	1,1
B	1,026	2,2	5,0	6,0	14,2
C	1,030	2,2	4,0	2,8	5,7
D	1,023	3,0	4,6	3,9	7,2
E	1,023	1,2	1,5	2,14	4,1
F	1,024	2,4	4,3	2,1	4,1

As shown in Table 1, 19-NA and 19-NE were formed in the specimens, the latter more efficiently. It is interesting to note that while the work published previously on in situ 19-demethylation was describing a rather minor process, with levels never reaching 1 ng/mL, the levels of 19-NA and 19-NE in the samples tested here went clearly above the threshold. Since in our hands the activity is not maintained once the specimens are frozen, the development of a reliable IRMS analysis was necessary to determine the origin of the urinary metabolites and to rule out the possibility of a rare but nevertheless occurring *in situ* reaction.

GC/C/IRMS Analysis: In Cologne, the isolation of 19-NA is carried out by a first HPLC separation utilising a dimethylaminopropyl column followed by a RPLC C_{18} to further separate 19-NA from androsterone[8]. We have adapted the method; a further purification step by semi-preparative reversed-phase HPLC (C_{18}) is added to the sample preparation to obtain more concentrated and less complex fractions, preventing interferences from the matrix (see experimental for conditions). The results thus obtained from the GC/C/IRMS analysis of low levels urinary 19-NA are reliable and reflect its synthetic or natural origin when compared to the values of androsterone, etiocholanolone and other reference urinary steroids not altered by the administration of the steroids.

Under such conditions, the GC/C/IRMS analysis of the 19-NA isolated from urine samples collected further to the ingestion of intact pig offal (concentration measured at 7,7 ng/mL), gave results consistent with the "natural" origin of the metabolites with a Delta ^{13}C value of -20,4. Table 2 lists the Delta ^{13}C values (expressed as $\delta^{13}C_{VPDB}{}^{0}/_{00}$) measured in routine confirmation analysis of 19-NA from an authentic standard and contained in a collection of urine samples provided following the ingestion of intact pig offal and utilized as negative control sample.

Table 2: Reproducibility of 19-NA Delta ^{13}C values in routine confirmation analysis

19-Norandrosterone sample (amount)	Delta ^{13}C ($\delta^{13}C_{VPDB}{}^{0}/_{00}$) Mean ± standard deviation
Authentic standard (10 – 20 ng)	-28,32 ± 0,76 (n = 13)
Negative control urine samples* (7,7 ng/mL)	-21,10 ± 0,58 (n = 7)

* Collection of urines following ingestion of intact pig: free and glucuroconjugates

We have also doped the negative control urine samples with an equivalent amount of synthetic 19-NA and as shown in Table 3, the Delta ^{13}C values thus measured reflect the proportionally depleted ^{13}C content.

Table 3: Variation of Delta ^{13}C values in mixed preparation

19-Norandrosterone sample (amount)	Delta ^{13}C ($\delta^{13}C_{VPDB}$ $^{0}/_{00}$)* Mean ± standard deviation
Authentic Standard (10 – 20 ng)	-27,83 ± 0,55 (n = 4)
Negative control urine (7,7 ng/mL)	-20,48 ± 0,58 (n = 3)
Mixture 50%	-23,86 ± 0,04 (n = 3)

The purification method was applied to routine confirmation of athlete' urine samples. The first athlete's sample in which *in situ* demethylation was confirmed by Cologne's newly developed GC/C/IRMS procedure was also analysed by us later; the Delta ^{13}C value of 19-NA was found at -20,4 (underivatized). The results of GC/C/IRMS analysis of 4 mL of urine containing 6,8 ng/mL of 19-NA are shown at Figure 1 as well as the GC/MS results (ion chromatograms and mass spectra of underivatized 19-NA).

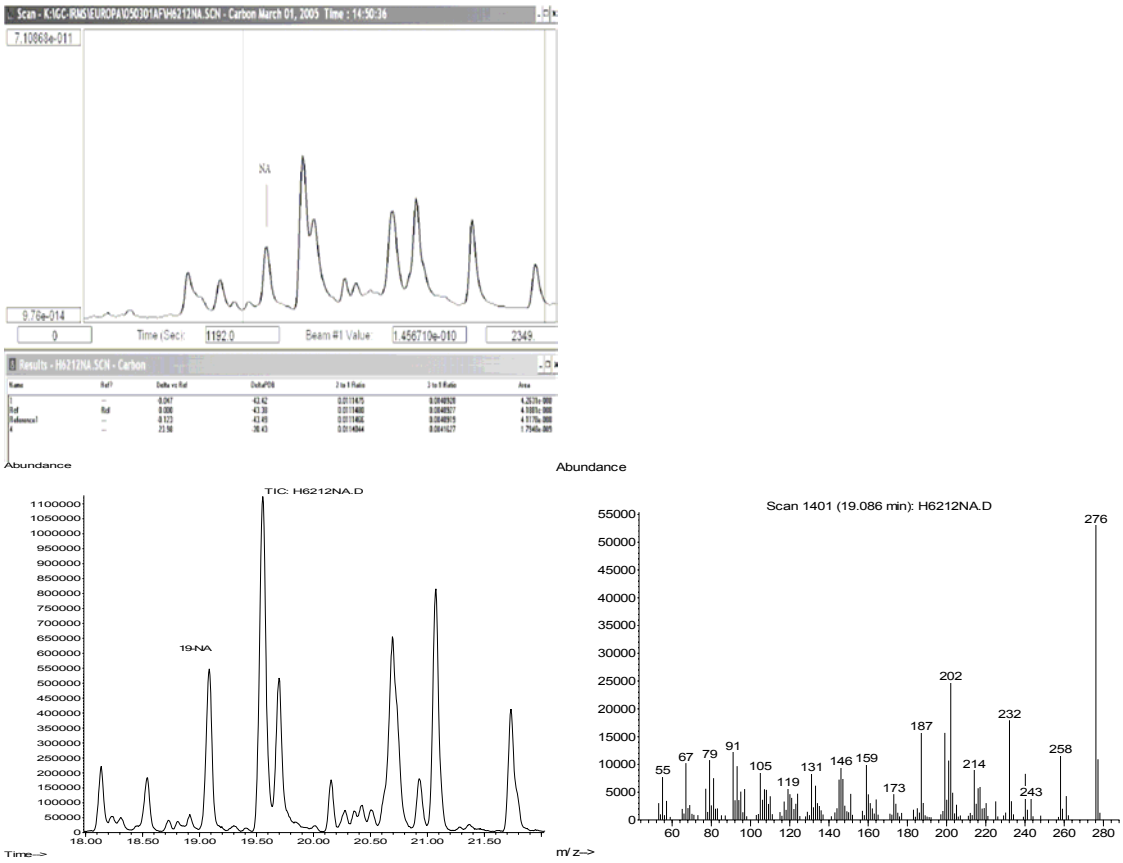

Figure 1: GC/C/IRMS (upper section) and GC/MS results of analyses of 19-NA in an "active" male athlete's sample (shared by the laboratory in Cologne).

Another example is shown in Figure 2; an athlete's sample found to contain 19-NA at a concentration of 8,5 ng/mL was purified and analysed by GC/C/IRMS. The results indicated its synthetic / exogenous origin, the Delta ^{13}C value was measured at -29.3 (Europa PDZ system).

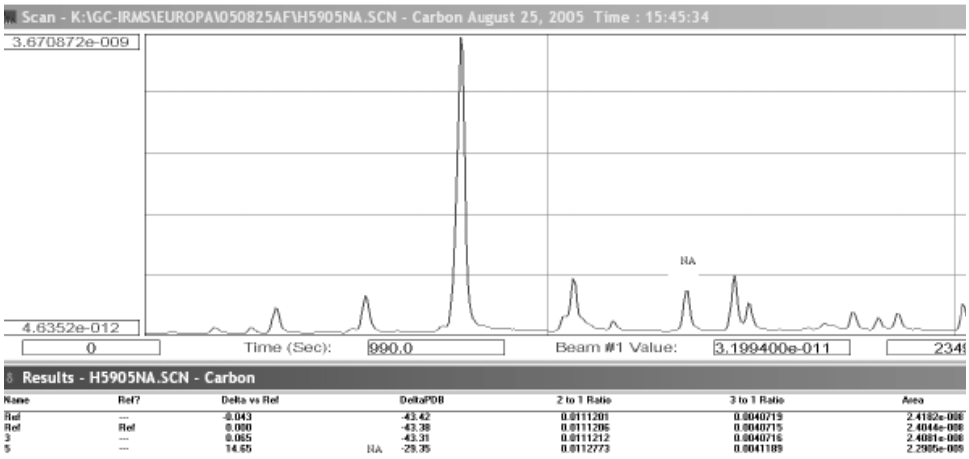

Figure 2: IR-Chromatogram and results of the GC/C/IRMS (Europa PDZ) analysis of 19-NA isolated from an athlete's urine sample (8,5 ng/mL).

As shown in Table 4, the GC/C/IRMS results indicated the endogenous origin of 19-NA, i.e. *in situ* demethylation in 10 out of 16,000 samples analysed in Montreal, most of them from the sport of tennis. The results from Cologne are also reported for comparison. The average concentration was low, 3,1 ng/mL and the criteria described in the technical note circulated in 2005, were met[10]. It is worth noticing that while the presence of a peak closely eluting with 19-NA (ion at m/z 405) in the GC/MS analysis of the TMS-derivatives, was often present in the "active" male athlete's samples detected in Montreal, two samples were found to contain exogenous 19-NA although the characteristics of active samples including that closely eluting peak were observed. In female samples in which *in situ* demethylation was detected following incubation, that peak was not present.

The GC/C/IRMS analysis confirmed the endogenous origin of 19-NA excreted at around 3,6 ng/mL in the urine samples of two female athletes as a result of pregnancy (indicated by the presence of high immuno-measured hCG levels). The exogenous origin of low levels of 19-NA, minor metabolite of norethisterone was also confirmed by their depleted ^{13}C content measured in two female athlete's samples.

Table 4: Results of routine GC/C/IRMS confirmation analysis of 19-NA identified in athlete's samples in Montréal and Cologne in the past two years[1].

Montreal (Total 16 000 samples) Period: 05-2004 to 03-2006 2 ng/mL ≤ 19-NA < 10 ng/mL	**Cologne (Total 20 000 samples)** Period: 2004 and 2005 2 ng/ml ≤ 19-NA < 20 ng/ml
Endogenous origin **Similar Delta ^{13}C Values of 19-NA and other urinary steroids**	
10 male samples ("activity") Tennis (7); Football (1); Canoe (2) Average concentration: 3.1 ng/mL 2 female samples (pregnancy) Average concentration: 3.6 ng/mL	2 female and 3 male samples (measured "activity") Weightlifting (1), gymnastic (1), skiing (1), athletics (1), soccer (1) Average concentration: 4.5 ng/mL 3 male samples (indications of "activity")* Rugby (1), fencing (1), soccer (1) Average concentration: 3.8 ng/mL 1 female sample (indications of "activity") Athletics Concentration: 3.5 ng/mL
Exogenous origin **Different Delta ^{13}C Values of 19-NA and other urinary steroids**	
2 female samples (birth control pill) Average concentration: 3.1 ng/mL 14 male samples Average concentration: 4.5 ng/mL	17 male samples, 3 female samples, 1 sample unknown Average concentrations: 8,3 / 9,5 ng/mL

The comparison of the Delta ^{13}C values of 19-NA and androsterone from athletes' samples received and analysed in Montreal is shown in Figure 3. In one group, the Delta ^{13}C values measured for androsterone and 19-norandrosterone were similar, indicating a common origin

[1] the female athlete's urine samples received in December 2004 were not analysed by GC/C/IRMS.

while in the second one, significant differences were noted, more depleted ^{13}C content being present in 19-NA supporting the exogenous origin (Delta ^{13}C values ranging from -27 to -32). Interestingly, in one specimen, the ^{13}C / ^{12}C value of 19-NA was less depleted (-19.5) than that of the other urinary steroids measured, androsterone, etiocholanolone and pregnanediol, all possessing Delta ^{13}C values around -24 ($\delta^{13}C_{VPDB}{}^{0}/_{00}$). The sample having been provided during routine controls and the data being kept on an anonymous basis, we have no knowledge of the athlete's diet, including the potential ingestion of a significant amount of intact pig's offal or the utilization of a norsteroid possessing a "natural" ^{13}C content.

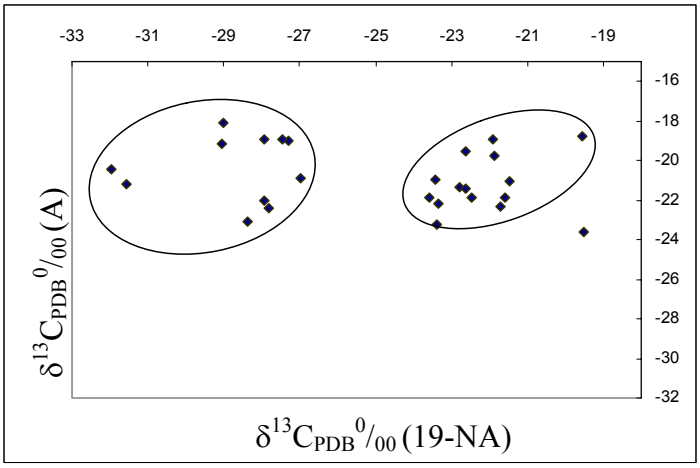

Figure 3: Comparison of Delta ^{13}C values of 19-NA and androsterone in routine confirmation of athlete's samples found to contain 19-NA in amounts ranging from 2 to 10 ng/mL (Montreal).

Analysis of urinary boldenone and metabolite:

From a case report presented in 1995 by Schänzer et al., we were alerted to the rare cases, exclusively males, involving the endogenous secretion of low levels of boldenone and of its metabolites, 17β-hydroxy-5β-androst-1-en-3-one and 3α-hydroxy-5β-androst-1-en-17-one[11]. Follow up with other urine samples was the only way to verify whether the excretion was systematic and natural or whether it had to be attributed to the administration of boldenone. The reversed-phase HPLC purification of 5 times 5 mL portions of the boldenone reference urine samples containing 10 ng/mL of boldenone and prepared according to the free and glucuroconjugated steroid extraction (UV detection at 245 nm; final volume 25 µL) is shown at Figure 4.

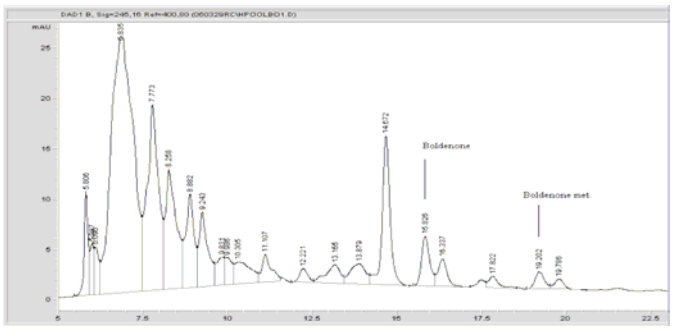

Figure 4: Reversed-phase HPLC chromatogram (Agilent Zorbax SB-C_{18} 5 µm; 9,4 x 250 mm; UV detection at 245 nm) of 5 x 5 mL of boldenone reference urine sample (10 ng/mL).

The fractions containing boldenone and its metabolite are analysed as such by GC/MS and GC/C/IRMS (Finnigan Delta^{plus} XP). The Delta ^{13}C values of boldenone, its metabolite, and other urinary steroids are summarised at Table 5.

Table 5: Results of GC/C/IRMS analysis of boldenone and its metabolite: authentic standards and isolated from the reference urine sample.

Fraction/Compound	**Delta ^{13}C Values ($\delta^{13}C_{PDB}$ $^{0}/_{00}$)**	
	Authentic Standard Direct / Following HPLC	Reference Urine (HPCL)
Boldenone (10 ng/mL) (14.6 to 17 min)	-33.62 / -33.53	-35.27 ± 0.16
Metabolite 1 (18.5 to 20.5 min)	-30.53 / -30.42	-34.84 ± 0.33
Androsterone Etiocholanolone Pgdiol		-20.15 ± 0.65 -22.10 ± 0.72 -20.18 ± 0.40

Conclusion: We have confirmed the usefulness of the GC/C/IRMS analysis of urinary extracts purified by semi-preparative reversed-phase HPLC in routine doping control test. The sample preparation certainly takes time and efforts but the reporting of an adverse finding is based upon clear and definitive evidence.

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