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RECENT ADVANCES IN DOPING ANALYSIS (6)

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Presence of norandrosterone in «normal» urine samples

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Nandrolone (19-nortestosterone or 19-nor-4-androsten-3-one-17α-olo) is one of the most largely used anabolic drug. As an example, doping controls found this substance in 212 cases during 1995, and in 232 cases during 1996 (1).

The metabolism of this drug, as well as the synthesis of metabolites, have been studied during the fifties and sixties (2). The main metabolite is norandrosterone (5α-estran-3α-ol-17-one) , and the identification of this substance in urine during doping controls has been always related with the abuse of nortestosterone.

During the last year, the extensive use of HR/SIR showed that very often signals due to norandrosterone in small amounts were present in urine samples. This observation prompted us to undertake a research which aimed to detect small amounts of norandrosterone currently present in "normal" urine samples.

In a first set of experiments, we compared the amount of norandrosterone present with a known amount (150 pg/ml of starting urine) of nortestosterone, added as internal standard. Of course, owing to the very low concentrations concerned, in most cases we observed that the peaks were interfered (see fig. 1). In separate GC\MS runs we monitored in one case the three ions in order to check the correct ratio among them and separately we compared the intensity of two ions, belonging one to norandrosterone and the other one to the standard (nortestosterone), in order to quantify the amount of norandrosterone.

Large errors are involved in these determinations and it is more correct to say quantitative "evaluation" instead of "determination". Nine urine samples (male athletes) were chosen in a large span of hormone content: the only criterion was a higher amount of androsterone than average.

Samples were processed according to the method currently used in the T/E ratio determination (see experimental): this procedure lowers the concentration of interfering substances, for instance the metabolite of vitamine E. The values found which refer to each sample are reported in table 1, third column. In the first column the laboratory numbers are reported.

TABLE 1

SAMPLE	ANDROSTERONE ng/ml	NORANDROSTERONE pg/ml (direct quantif.)	NORANDROSTERONE pg/ml (HPLC purif.) x2
P7	3000	48	36
Q12	3350	78	30
P48	3700	35	110
Q4	5200	40	64
Q9	5200	108	96
Q3	6300	116	100
P99	6600	59	135
J299	10300	155	96
Q11	12000	126	68

All urines tested contain norandrosterone in small amounts, the concentration not exceeding 160 pg/ml.

The presence of norandrosterone has been confirmed in all cases through an independent experiment, i.e. by a purification of the same samples by HPLC, and determining the presence of norandrosterone by GC/MS/HRSIR. With this procedure we obtained very often perfectly clean traces (see fig.2), and in this way we had not doubts about the presence of nor androsterone. In contrast, the quantitative evaluation, performed by addition of nortestosterone as standard after the HPLC purification, was not accurate, since in our experience, the loss associated with the chromatographic run can be roughly evaluated as 50%.

Taking into account of the above mentioned loss and by correcting the results obtained, the amount of nandrolone metabolite is reported in table 1, fourth column: in all samples examined, the presence of the substance was confirmed.

In a second set of experiments we took nine samples from male athletes and nine from female athletes. Criteria of choice were the same as stated above: androsterone content higher than the average. The feminine urine samples were preliminary examined if they contained norethisterone metabolites, as residues of anti-contraceptive pills. The method was that currently used in the laboratory.

Here we considered also the signals due to noretiocholanone (supposed to be the second metabolite of nortestosterone) and a rough estimate of this substance was also made. In most cases nor etiocholanone was found in an amount lower than that of norandrosterone, but not negligible. Again we used both methods: same samples were processed according the modified screening IV and according the method of purification by preparative HPLC.

Results are reported in Table 2: norandrosterone and noretiocholanone are indicated as **M1** and **M2** respectively

TABLE 2

MASCULINE SAMPLES

SAMPLE	ANDROSTERONE ng/ml	NORANDROST. (**M1**) pg/ml (direct quantific.)	NANDR. METAB. pg/ml (HPLC purif.) x 2	
			M1	**M2**
P46	2700	130	56	traces
S26	3300	130	110	80
Q7	3700	40	20	<10
Q5	4200	90	65	40
J293	4200	150	80	70
S28	8000	150	250	105
J285	8100	110	110	80
J287	8200	100	60	20
S30	8200	n.q. (interfered)	100	50

FEMININE SAMPLES

SAMPLE	ANDROSTERONE ng/ml	NORANDROST. (**M1**) pg/ml (direct quantif.)	NANDR.METAB. pg/ml (HPLC purif.) x2	
			M1	**M2**
A61	970	100	40	75
A52	1100	300	320	70
A67	1300	100	160	10

A60	2000	n.q.	20	20
A97	2100	90	60	<10
A88	2100	70	140	30
A69	2250	n.q.	110	130
A96	2650	280 (?interfered)	55	13
A37	2677	n.q.	50	<10

n.q. = not quantifiable

These results strongly suggest that norandrosterone and nor etiocholanone are endogenous steroids. This is not surprising, since it is possible to envisage different pathways leading to these substances. For example, demethylation at C-6 occurs during the conversion of androstenedione into oestrone (3): deputy enzymes could be not highly specific. Also, cholesterol biosynthesis involves different steps in which a demethylation occurs at C-14 (4); the same enzymes could be not sufficiently specific to avoid the occurrence of a demethylation at C-6, suffered by substrates which are part of the catabolic cascade of steroids.

The limited number of data collected here prevent any conclusion about the «physiological» or «normal» level of these substances in urine samples of healthy subjects. However it should be not difficult to perform a multi-laboratory study to find these data: of course it should be necessary to have a correct protocol to quantify such a small amount. Researches based on the use of deuterated norandrosterone are in progress in our laboratory.

NOTES

(1) Statistics 1995 and 1996 of the IOC Accredited Laboratories

(2) L.L.Engel, J.Alexander, M.G.Weeler J.Biol. Chem. 231,158-165 (1958) ; D. Kupfer, E. Forchielli, R.I. Dorfman J.Org. Chem. 25, 1674-1675 (1960)

(3) M.Akhtar,S.J.M.Shinner, Biochem. J. 109, 318 (1968), and references therein.

(4) K.Alexander,M.Akhtar,R.B.Boar,J.F.Mcghie,D.H.R.Barton J.C.S.Chem.Comm. 383 (1972)

EXPERIMENTAL

Modified screening IV (purified conjugate fraction); GC/MS/HRSIR

a) ***Sample preparation:*** Urine (5ml) was passed through a Sep-pak C18 cartridge (pre-washed with MeOH and water), and after washing with H_2O (2 ml) the cartridge was eluted with MeOH (3ml). Methanol was removed with a stream of N_2 at 40°C, and the residue dissolved in phosphate buffer pH 7.4 (1.5 ml), and extracted with peroxyde free diethyl ether (5 ml). The ethereal solution was discarted, the aqueous soln flushed with N_2, and the internal standard added (nortestosterone, 750 pg). The soln was incubated at 55° C for 1 h with β-glucuronidase (50 µl), carbonate buffer (pH 9) was added (1 ml), and the soln extracted with n-pentane (5 ml). The organic phase was dried under a stream of N_2 at 40° C and the residue dried in a dessiccator and derivatized with MSTFA/NH4I/DTE 1000:2:4 (20 µl) at 65° C for 40 min.

b) ***GC/MS*****:** HP-5 cross linked capillary column, 18 m lenght, I.D. 0.2 mm, film thickness 0.33 µm: constant flow 0.8 ml/min. Temperature program: T_0 = 220° C, time 1 = 2 min, rate 7° C/min; T_1 = 300° C, time 2 = 7 min. Injector temperature = 300° C.

c) ***HRSIR:*** AUTOSPEC TOF, acquisition mode SIR. Qualitative Analysis: resolution 3500 (5% valley definition) acquisition start time 6:30 min.: ions monitored 315.21, 405.26, 420.29 m/z; Quantitative analysis: resolution 4500 (5% valley); ions monitored 405.21 (norandrosterone), 418.27 (nortestosterone). GC Rt of norandrosterone (min): 6.52; Rt of nortestosterone (int. std): 8.49 min.

Sample preparation and HPLC purification procedure

a) ***Sample preparation:*** Urine (10 ml) was processed as stated above and the n-pentane extract was dried in the same way under a stream of N_2. The residue was dissolved in 25 µl of MeOH, Phenanthridine (20ng) was added as HPLC run marker, and the soln was injected in HPLC.

b) ***HPLC purification:*** Column Whatman Partisphere C18, 25 cm length, I.D. 4.6 mm, particle size 5 um. Pre-column Waters C18. Flow: 1ml/min. Eluent: gradient methanol-water. Gradient programme:

Time(min)	Water	Methanol
0.01	55%	45%
5.00	37%	63%
10.00	23%	77%
15.00	11%	89%
20.00	5%	95%
25.00	0%	100%
30.00	55%	45%

Detector HP 1040, marker Rt 12.4 min: Fractions collected: 13.5-14.1 min noretiocholanone; 14.1-14.9 norandrosterone. Solvent was removed with a stream of N_2, and the standard added to the residue. This material was derivatized as reported above.

c) ***GC/MS/HRSIR*:** same conditions as stated above

FIG.1: sample A97 (lab's code), norandrosterone Rt = 6:52, upper trace ion 405, lower trace ion 420.

FIG.2: sample A97 (lab's code), after HPLC purification, norandrosterone Rt = 6:52; upper trace ion 405; middle trace ion 420; lower trace ion 315.

File:SCRNORA97 #1-321 Acq:20-FEB-1998 16:02:07 GC EI+ Voltage SIR AutoSpecTOF
405.2645 Exp:NORASIR
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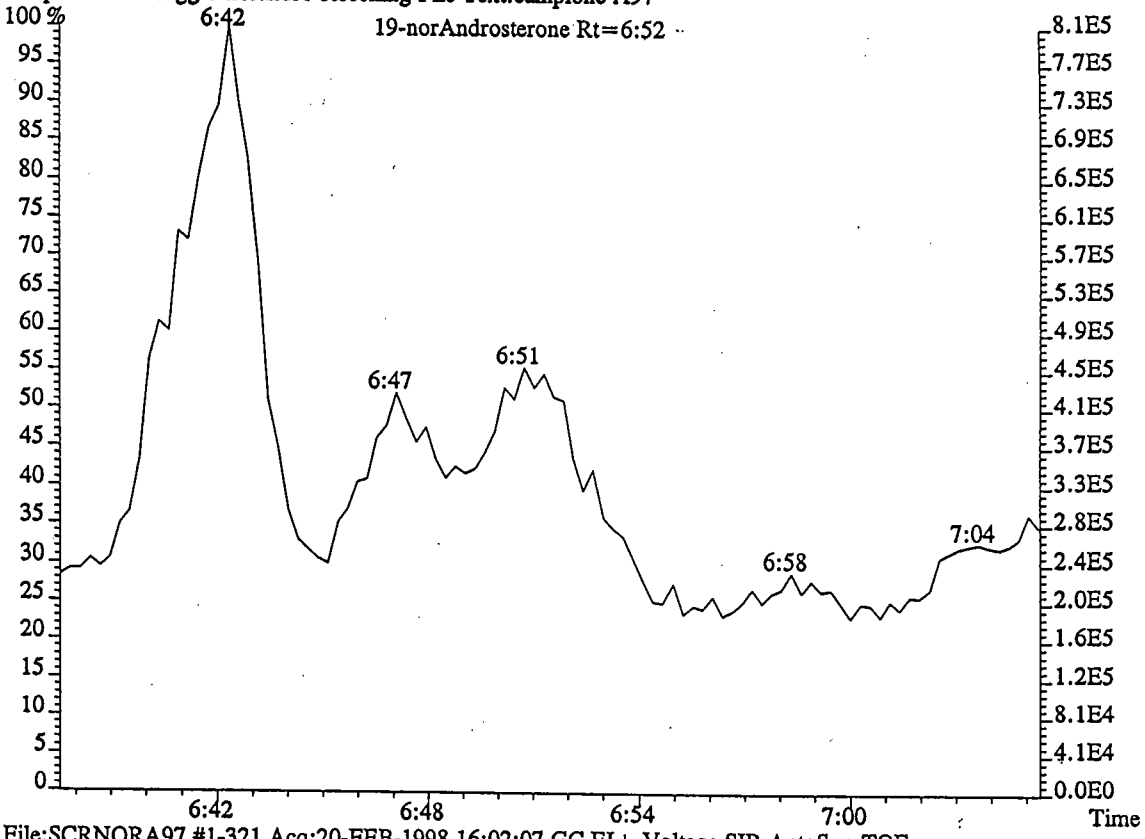

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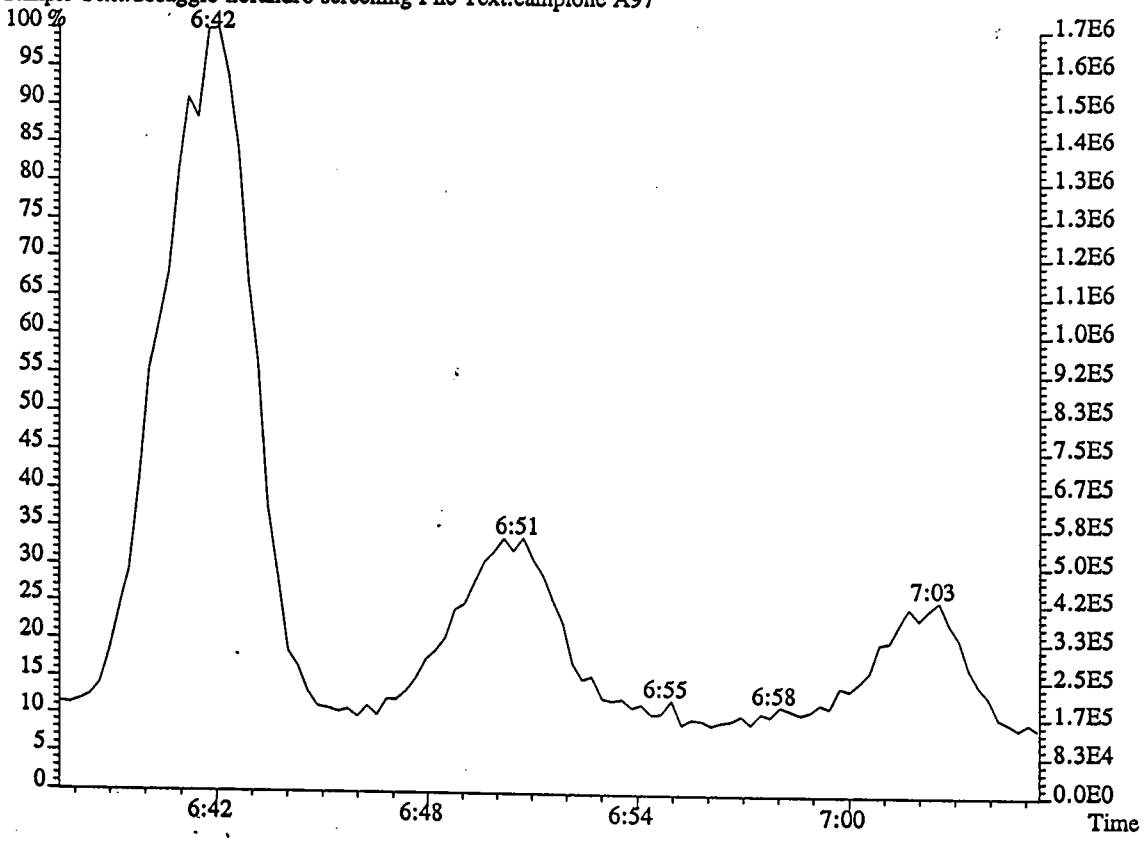
420.2880 Exp:NORASIR
Sample Text:dosaggio norandro screening File Text:campione A97



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405.2645 SMO(2,3) BSUB(128,15,-3.0) Exp:NANDROLONE Noise:2303
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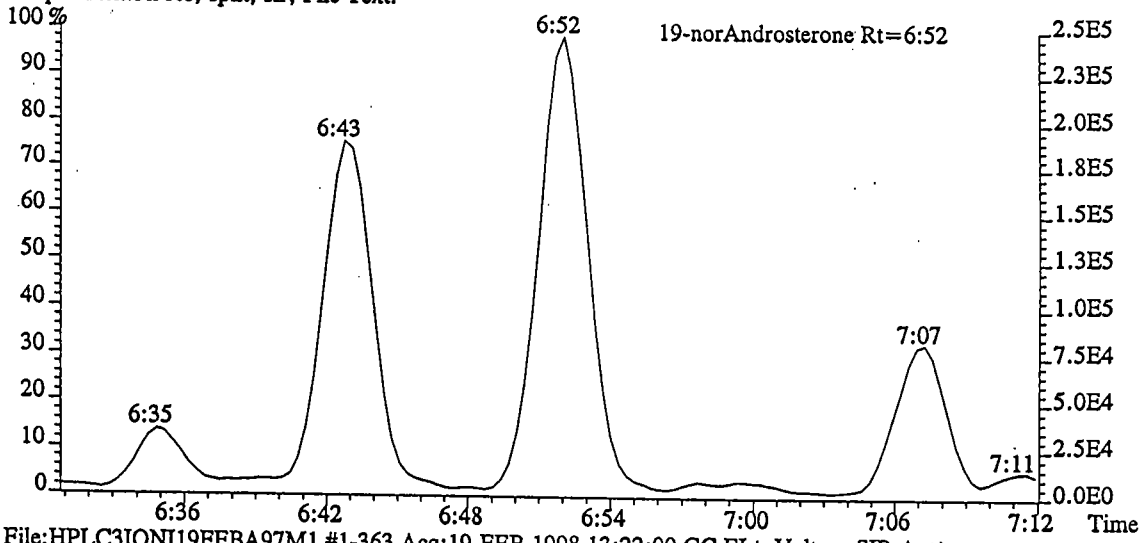

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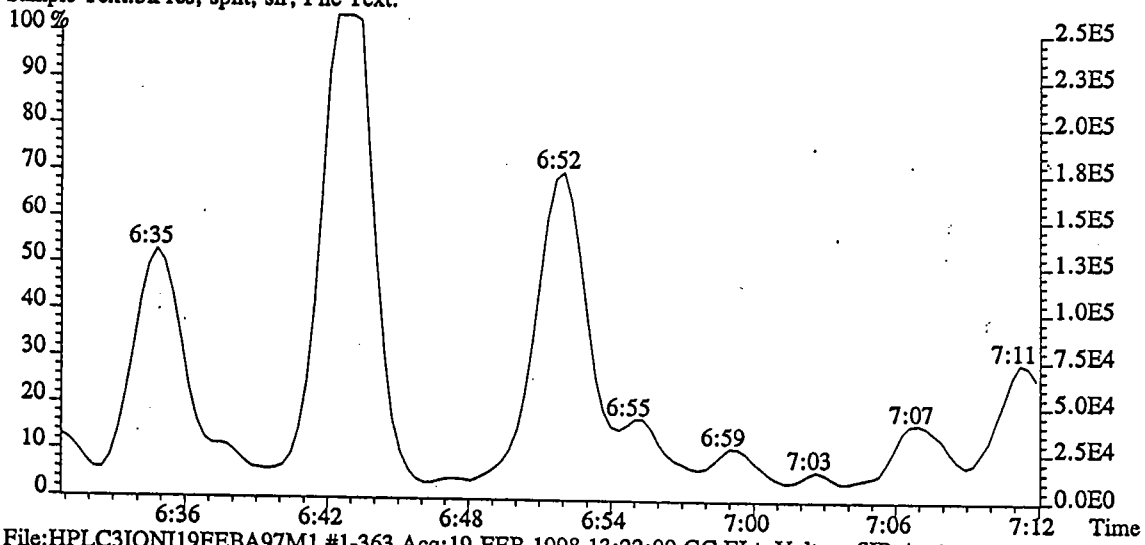

File:HPLC3IONI19FEBA97M1 #1-363 Acq:19-FEB-1998 13:22:00 GC EI+ Voltage SIR Aut>
315.2144 SMO(2,3) BSUB(128,15,-3.0) Exp:NANDROLONE Noise:2919
Sample Text:5k res, split, sir; File Text: