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Determination of nandrolone and metabolites in urine samples from sedentary persons and sportsmen☆

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Abstract

Metabolites of nandrolone were determined in the urine of several sportsmen, sedentary and post-menopausal women by capillary gas chromatography–mass spectrometry quadrupole (GC–MS) and capillary gas chromatography mass–mass spectrometry ion trap (GC–MS–MS) methods. The method employed was GC–EI-MS with 17α-methyltestosterone as internal standard with ethyl ether extraction prior to selected ion monitoring of the bis(trimethylsilyl) ethers at ion masses m/z 405 and 420 for the nandrolone metabolites, and 418 and 403 for nandrolone derivative. Recovery for nandrolone, 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) was 97.20, 94.17 and 95.54%, respectively. Detection limits for nandrolone, 19-NA and 19-NE were 0.03, 0.01 and 0.06 ng/ml. Metabolites of nandrolone (19-NA and 19-NE) were found in 12.5% ($n=40$) of sportsmen and 40% ($n=10$) of post-menopausal women.

Keywords: Nandrolone; 19-Norandrosterone; 19-Noretiocholanolone

1. Introduction

Nandrolone is a substance included in Class C of prohibited substances in Appendix A of the Olympic Movement Anti-Doping Code (OMAC) [1]. In the human body, nandrolone and its metabolites may be of exogenous and endogenous origin.

Exogenous origin is due to administration of nandrolone and derivatives (nandrolone decanoate, phenylpropionate, propionate, etc.), ingestion of contraceptive steroids [2], ingestion of contaminated food (steroids are used in animal feed to improve the nitrogen balance and to accelerate the growth rate of cattle for slaughter) or the ingestion of special nutrition for bodybuilders. The possibility of analytical interference due to the consumption of hormone-contaminated meat in urine doping control was

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first described by Debruyckere et al. [3] and was confirmed in 1991 [4]; this possibility was evidenced by Debruyckere et al. in 1992 [5] and recently reported by Le Bizec [6].

The endogenous origin of nandrolone is a controversial topic in doping control: firstly, because its urine detection is based on the presence of the two main metabolites, namely 19-norandrosterone (3α-hydroxy-5α-estran-17-one; 19-NA), and 19-noretiocholanolone (3α-hydroxy-5β-estran-17-one; 19-NE) [3,7,8], secondly, because low-dose oral administration of 19-norandrostenedione (4-estrene-3,17-dione) can produce similar urinary metabolites [8]; and thirdly, because endogenous production of nandrolone by aromatase-rich tissues has been reported [9].

A result is positive if the concentration of 19-NA in urine is higher than 2 ng/ml for men and 5 ng/ml for women. However, the origin of these nandrolone metabolites in untreated men has not yet been elucidated. For instance, Debruyckere et al. [3] reported three male volunteers with basal urinary 19-NA concentrations between 9 and 37 ng/ml.

Research in 1982 [10] and 1988 [11] showed that nandrolone and its metabolites exist naturally as intermediates during production of estrogenic compounds. Le Bizec et al. [7] recently published results showing than more than 50% of individuals tested voided urine containing 19-NA and 19-NE. Concerning the IOC limit level, work by Debruyckere et al. [3] has shown that 2 ng per ml is a gross underestimate of the concentration of nortestosterone metabolites which occur naturally in urine. Debruyckere et al. found that more than 1% of all individuals tested produced natural urinary concentrations of nortestosterone metabolites above the 2-ng/ml level. One individual provided a urine sample with 37 ng/ml of nortestosterone metabolites, 18.5 times greater than the level specified by the IOC. The concentration of metabolites also is not constant over time, being affected by external stimuli. This was demonstrated by Le Bizec et al. [7] who demonstrated that exercise, prior to sampling, increased 19-NA urinary concentrations by a factor of 4.

The starting point for the method proposed in this work was an IOC reaccreditation procedure (Laboratorio Control del Dopaje, Madrid). In this work, an GC–EI-MS specific study on the detection and quantitation of 19-NA and 19-NE and the use of GC–MS–MS ion trap for identification of positive samples is described. In the present study, nandrolone metabolites were determined in urine of post-menopausal women, sportsmen, sportswomen, and the family of a nandrolone positive sportsman. This study attempts to show that there can be situations where nandrolone can be produced endogenously and/or through food, though how this happens is not yet clear. One of the purposes of this work was to employ the GC–MS method for the detection of nandrolone metabolites in prescribed sports, and avoid the possibility of false positive results in antidoping control.

2. Experimental

2.1. Urine collection

A total of ten post-menopausal women, 36 healthy sportsmen and four sportswomen (marathon, basketball, triathlon) and the family (father, mother and brother) of one nandrolone positive sportsman were analysed using their first urine of the day. Urine samples of the nandrolone positive man and his brother were obtained before and after 2 h of exercise. The fraction volumes were measured and an aliquot was stored at −4°C until analysis.The population under study was divided into three groups: (A) post-menopausal women: age 61±6 years, weight 68±13 kg, height 1.6±0.5 m, not receiving medical treatment during the investigation; (B) sportsmen: basketball players belonging to the Spanish professional league (ACB): age 30±5 years, weight 92±10 kg, height 2.0±0.7 m, and students of the Faculty of Sciences of Sport: age 21±2 years, weight 72±7 kg, height 1.8±0.5 m; and (C) the sportman's family: positive sportsman: age 21 years, weight 86 kg; brother: aged 24, weight 80 kg, height 1.92 m.

2.2. Materials

Nandrolone, 17α-methyltestosterone (internal standard) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were purchased from Sigma–

Aldrich (Steinheim, Germany), 19-norandrosterone and 19-noretiocholanolone were kindly donated by Consejo Superior de Deportes (C.S.D.), β-glucuronidase from *Escherichia coli* K 12 (200 IU/ml) was supplied by Boehringer Mannheim (Mannheim, Germany), dithioerythritol (2,3-dihydroxybutane-1,4-dithiol) was from Serva (Feinbiochemica, Heidelberg, Germany), and ammonium iodure was purchased from Panreac (Barcelona, Spain). Organic solvents and other usual chemicals were obtained from common chemical suppliers, were of analytical grade and were used without further treatment.

2.3. Gas chromatography–mass spectrometry

The GC–MSD system (Model 5972 mass-selective detector combined with a Model 5890 Series II gas chromatograph, Hewlett-Packard) was used in both scan and selected-ion-monitoring modes. The gas chromatograph was equipped with a 25 m×0.25 mm×0.25 μm capillary column coated by cross-linked 5% phenyl-methyl silicone fluid. The carrier gas was helium at a flow-rate of 1 ml/min. The splitless method of injection was used. The oven was maintained at 100°C for 1.5 min, then increased to 260°C at a rate of 10°C/min, maintained for 5 min, and finally increased to 300°C at a rate of 30°C/min with 5 min for the final ramp. The electronic beam energy was set at 70 eV in the electronic impact (EI) mode.

To confirm the results, the conditions of EI and mass–mass (MS–MS) were performed with a Varian 3800 gas chromatograph and Saturn 2000 ion trap detector coupled to the Saturn GC–MS workstation data processing system.

A capillary cross-linked methyl silicone (15 m× 0.2 mm I.D.×0.1 μm film thickness) column was used in the gas chromatograph. Ultra-high-purity helium at a flow-rate of 1 ml/min was used as the carrier gas. A splitless injection mode was used with the injector set at 280°C. The initial oven temperature was set at 120°C (for 2 min) and programmed to increase at 20°C/min to 250°C (for 4 min), then the temperature was increased at 20°C/min and held for 5 min. The detector temperature was 200°C (trap), 50°C (manifold) and 280°C (transfer line). The MS–MS conditions are summarized in Table 1.

2.4. Calibrations, recovery and reproducibility

The limits of detection and quantitation for different levels of the regression curve with standards and spiked urine (2 ml) were calculated with 0.05-, 0.1-, 0.5- and 1-ppb levels through partial least-square methods. The working calibration curve levels were 1, 6, 12, 25 and 50 ng/ml for nandrolone and its metabolites, and the graphs were constructed by plotting the peak area ratios of the analyte target quantitate ion (405 for 19-NA and 19-NE, 403 for nandrolone) taking the precaution of using the SIM mode [12] for detection; the MS–MS method was used to confirm the results.

Recovery was calculated by adding 50 ng/ml of the compounds analysed in 2 ml of blank solution of human urine with seven replicates.

The reproducibility of this method was determined by injection of seven replicates of 2 ml of urine spiked with 50 ng/ml of the analytes.

2.5. Sample preparation

The standards were added to vials with 250 ppb of 17α-methyltestosterone as internal standard, and the solvent was evaporated to dryness under a nitrogen stream.

After addition of internal standard to a 2-ml urine sample, 125 μl of phosphate buffer (pH 7, 0.2 *M*) was added and the urine was hydrolysed with 50 μl

Table 1
Experimental conditions of MS–MS method

Compound	Relative Retention Time (RRT)	Ion	RF	Voltage
19-NA	0.86	405	108	80
19-NE	0.88	405	125	80
Nandrolone	0.94	418	100	66
I.S.	1	–	–	–

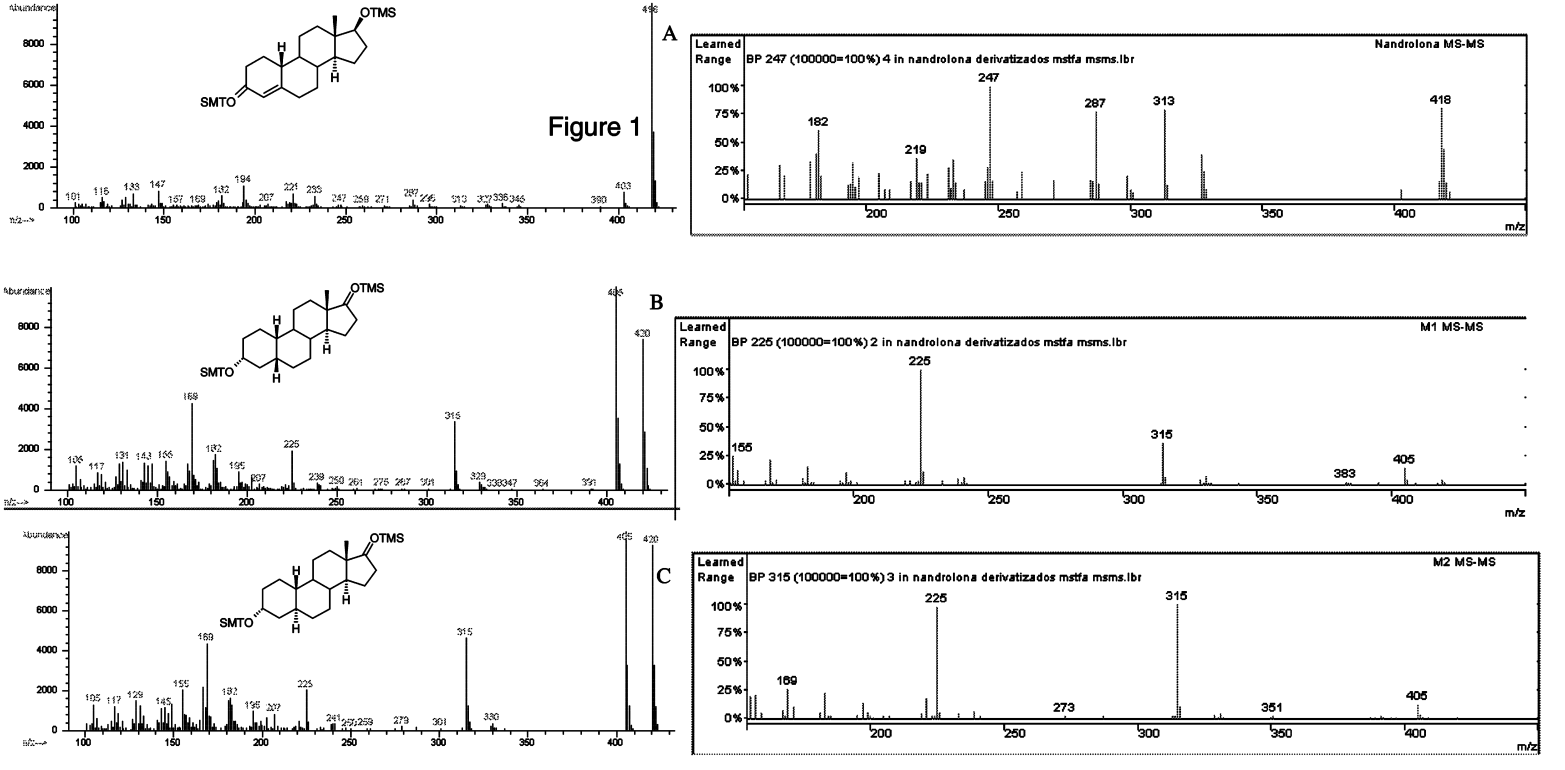

Fig. 1. (A) Nandrolone, (B) 19-norandrosterone, (C) 19-noretiocholanolone; left (TIC spectra), right (MS/MS spectra).

Table 2
Limit of detection (LOD) and quantitation (LOQ) for the analytes

Substance	LOD (ng/ml)	LOQ (ng/ml)
Nandrolone	0.03	0.1
19-Norandrosterone	0.01	0.03
19-Noretiocholanolone	0.06	0.2

of β-glucuronidase (1 h, 50°C). The urinary extract was centrifuged at 2000 *g* for 5 min to eliminate solid residues. Then the sample was adjusted to pH 9 with potassium carbonate buffer (7%) and extracted with 2 ml of diethyl ether during 15 min in a mixer at 110 rpm. The organic phase was then evaporated to dryness under a gentle nitrogen stream.

2.6. Derivatization of urine extract

The dry steroid residue was derivatized with 50 μl of MSTFA/NH4I/dithioerythritol (1000:2:3, v/w/w) solution at 60°C for 15 min to generate the TMS ether derivatives. A 1-μl sample of the liquid residue was injected in splitless mode.

3. Results and discussion

3.1. Mass spectra and mass–mass spectra

The total ion current spectra for the TMS derivatives and the MS–MS spectra are shown in Fig. 1. The interesting peaks for analytical purposes are the 420, 405 and 315 for the metabolites, in SIM mode for identification (405 for quantitation), and 418, 403 and 313 for nandrolone (403 for quantitation).

3.2. Detection limits, recovery and reproducibility

Detections limits are shown in Table 2.

The results for recovery were 94.17±8.3%, 95.54±8.4%, and 97.72±4.3% for 19-NA, 19-NE and nandrolone, respectively, for seven replicates.

Concerning reproducibility, the RSD were 5.1, 5.7 and 6.4% for 19-NA, 19-NE and nandrolone, respectively, for seven replicates.

3.3. Detection of 19-norandrosterone and 19-noretiocholanolone in urine samples

Metabolites of nandrolone were found in some of the urine samples of the population under study but not in all.

In post-menopausal women, results were as follows: one had significant levels of metabolites (Fig. 2), while three showed a suspected signal of two metabolites which was confirmed by MS–MS (Table 3). In all four samples the 19-NA concentration was higher than 19-NE.

In sportsmen, only four of 36 urine samples contained significant 19-NA and 19-NE levels, 19-NA being the major metabolite. 19-NA was detected in one of the four samples from sportswomen, whereas 19-NE was undetectable (Fig. 3). It may be normal that 19-NE does not appear in the urine of women (S-W 2) positive for 19-NA, and the suspicious peak could belong to other isomers of this metabolite (3β-hydroxy-5α-estran-17-one, 3β-hydroxy-5β-estran-17-one): this needs to be confirmed.

Urine levels of 19-NA and 19-NE were found in all tested members of the family of a positive nandrolone sportsman (father, mother, sportsman,

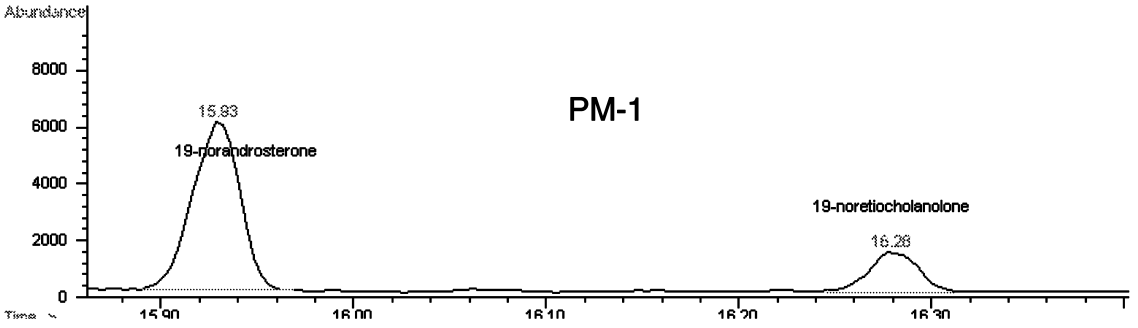

Fig. 2. Woman no. 1 with two metabolites.

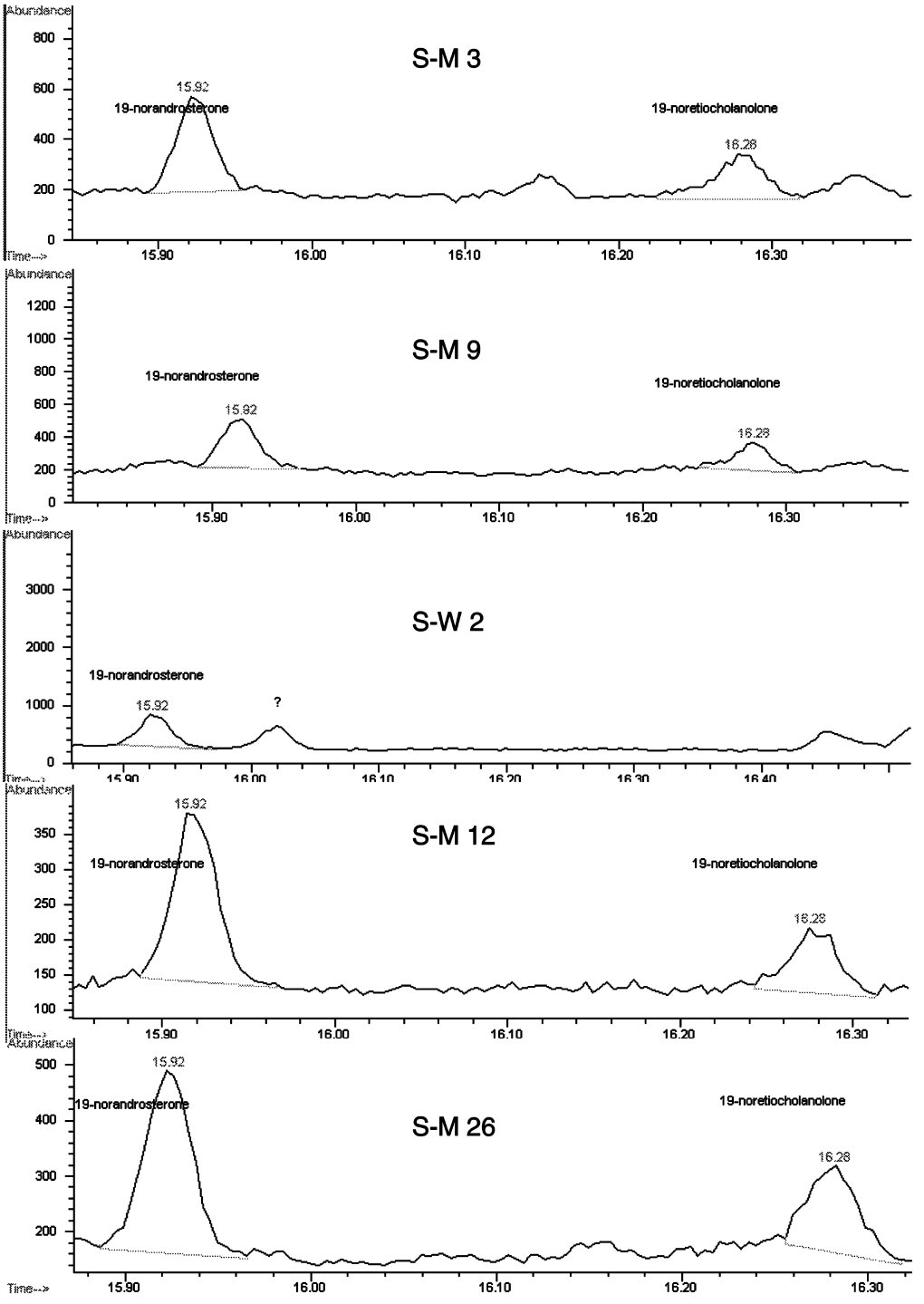

Fig. 3. Chromatograms showing 19-NA and 19-NE peaks for sportmen (S-M) and sportswomen (S-W).

Table 3
Presence of metabolites in volunteers

Sample reference	19-NA		19-NE	
	Result	Conc. (ng/ml)	Result	Conc. (ng/ml)
P-M 1	++	22	++	6
P-M 3	+	–	+	–
P-M 4	+	–	n.m.	–
P-M 7	+	–	+	–
S-M 3	++	14	++	6.5
S-M 9	++	8	+	–
S-W 2	++	4	n.m.	–
S-M 12	++	6	+	–
S-M 26	++	5	+	–

+, concentration between LOD and LOQ; ++, concentration higher than LOQ; n.m., not measurable; P-M, post-menopausal; S-M, sportsman; S-W, sportswoman.

brother), in a 2:1 19-NA/19-NE ratio, as has been described by others [7] in the urine of sportsmen. However, after exercise, the proportion of 19-NE was greater than that of 19-NA (Fig. 4), and the proportion of 19-NA to 19-NE was normally inverted in these samples. Work to elucidate this fact has been started.

A possible strategy to elucidate the endogenous origin of these substances may be to consider diet, levels of aromatase that can rupture normal metabolic routes, and correlation with other possible steroids (androsterone, androstenedione, etc.).

From the results obtained, presented in Table 3, our study shows a higher percentage of detectable

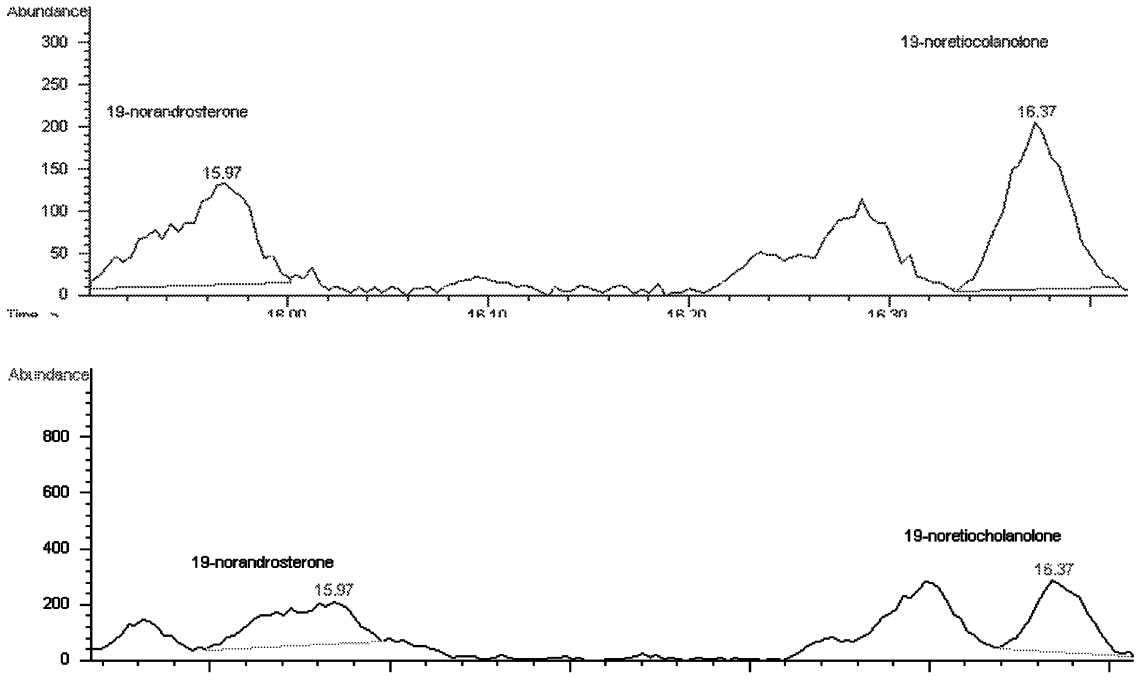

Fig. 4. Chromatograms that show inversion of the ratios 19-NA/19-NE in samples after exercise in sportsman and brother.

nandrolone metabolites in women than in men. This fact may be due to the higher levels in women of aromatase, which can promote higher endogenous nandrolone production [8].

Finally, ours results confirm those of Dehennin [8], alerting sport authorities to possible reasons for positive results.

Acknowledgements

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