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Detection and determination of anabolic steroids in nutritional supplements

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Abstract

A method is described for the determination of anabolic steroids including testosterone, 19-nor-4-androstene-3,17-dione, 4-androstene-3,17-dione and nandrolone in food supplements. Initial clean-up is done by HPLC followed by determination with GC/MS. A 'contaminated' food supplement was analysed and appeared to contain 19-nor-4-androstene-3,17-dione and 4-androstene-3,17-dione. One capsule of this nutritional supplement was ingested by five male volunteers. Urine samples were collected and analysed by GC/MS and GC/MS-MS. Neither the ratio testosterone/epitestosterone, nor the ratio androstenedione/epitestosterone increased significantly. Concentrations above 2 ng/ml for norandrosterone, the major metabolite of nandrolone, were detected until 48–144 h after ingestion of the food supplement.

Keywords: Nutritional supplement; Anabolic steroids; Norandrostenedione; Detection; Excretion

1. Introduction

The use of nutritional supplements in sports is widespread. Even for professional sportsmen the temptation for experimenting with these supplements is big. A strong indication of this trend comes from the increased sales of herbal medicines in the US [1]. The ease of availability stimulates the use of these products. On the Internet numerous sites can be found where one can order all kinds of 'natural' products. Moreover manufacturers claim the necessity of food supplements in order to perform better and to be competitive.

Creatine is perhaps one of the most used supplements at the moment. Currently it is legal and its use by athletes is not considered as doping. Caffeine can also be found in several supplements (e.g. guarana containing supplements), although according to IOC doping regulations the

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urinary concentration of caffeine may not exceed 12 ppm. Ephedrine alkaloids are also frequently found in herbal supplements (e.g. Ma Huang). These products can lead to deleterious side effects when not used properly [2].

Nowadays even anabolic steroids are available as 'nutritional' supplements. Moreover it was shown that in some cases supplements contained banned substances not indicated on the label [3].

In this study, a method to determine several anabolic steroids in food supplements is presented. Urinary concentrations of endogenous steroids and the major metabolites of nandrolone, norandrosterone and noretiocholanolone, are determined in five male volunteers after the administration of a 'contaminated' food supplement.

2. Experimental

2.1. Reagents

Nandrolone, testosterone, 19-nor-4-androstene-3,17-dione, 4-androstene-3,17-dione and 17α-methyltestosterone were purchased from Sigma (St. Louis, MO). Norandrosterone and noretiocholanolone were obtained from Schering (Berlin, Germany). The enzyme preparation β-glucuronidase type HP-2 from Helix pomatia (100000 U/ml β-glucuronidase and 5000 U/ml sulfatase) was obtained from Sigma. *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was obtained from Macherey-Nagel (Düren, Germany). HPLC grade acetonitrile was from Acros (Geel, Belgium). All other chemicals were of analytical grade.

The food supplement was from US origin. The labelled composition was as follows: seven capsules contain: garcinia cambogia 2000 mg, L-carnitine 1100 mg, guarana extract 1025 mg, citrus aurantium extract 300 mg, L-phenylanaline 300 mg, dandelion root 250 mg, choline bitratrate 200 mg, cayenne powder 60 mg, vanadyl sulfate 15 mg and vitamin B6 10 mg and 'much more'. The manufacturer recommended seven capsules on a daily base.

Steroid free urine was obtained by pouring urine over a glass column filled with XAD-2. The absence of all endogenous steroids in the urine was determined by GC/MS analysis prior to use.

Table 1
GC relative retention time (RRT) and monitored m/z-values for trimethylsilylated[a] endogenous steroids

Steroid	RRT	m/z
Androsterone	0.745	419–434
Etiocholanolone	0.756	419–434
5α-Androstane-3α,17β-diol	0.766	241
5β-Androstane-3α,17β-diol	0.773	241
5α-Androstane-3β,17β-diol	0.851	421
Epitestosterone	0.851	432–417
5α-Dihydrotestosterone	0.865	434
4-Androstene-3,17-dione	0.884	430–415
Testosterone	0.901	432–417
11β-Hydroxy-androsterone	0.919	522
11β-Hydroxy-etiocholanolone	0.930	522
17α-Methyltestosterone (IS)	1.000	446–301

[a] ketosteroids as TMS-enolethers.

2.2. Equipment

2.2.1. HPLC

The HPLC system consisted of a SP 8800 ternary pump (Spectra-Physics, California, USA), SP 8880 autosampler and a Spectra Focus forward optical scanning detector (UV3000), set at a wavelength range of 220–360 nm. A 100 × 3 mm ID column packed with octadecyl silica (5 μm Nucleosil, Chrompack, Antwerp, Belgium) with an appropriate guard column was used. The column was held at room temperature. The loop volume was 20 μl. The mobile phase consisted of acetonitrile and acetic acid (1% in H_2O) at a flow rate of 1 ml/min. The gradient program was as follows: initial acetonitrile was 2% for 4 min, increased to 32% in 6 min, finally to 45% in 6.5 min, hold for 4 min. Afterwards the column was equilibrated for 10 min with 2% acetonitrile.

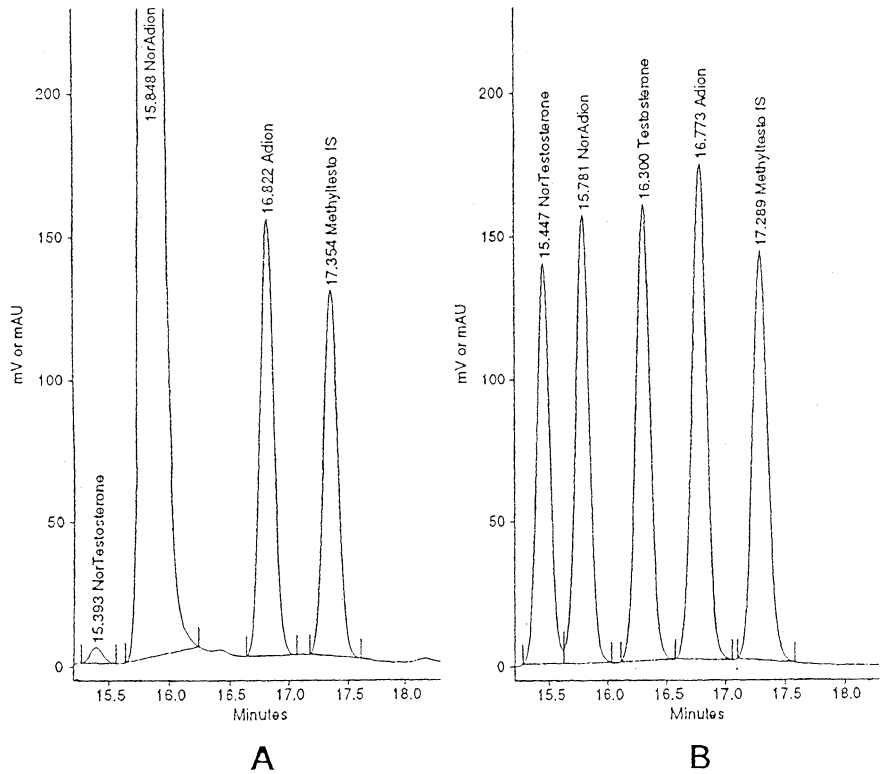

Fig. 1. (A) A part of the HPLC chromatogram of the extract of the food supplement at a scan wavelength of 240 nm. (B) A part of the HPLC chromatogram of the references at a scan wavelength of 240 nm.

2.3. GC/MS

2.3.1. Determination of anabolic steroids in food supplements

The GC/MS analyses were conducted in full scan mode on a HP (Hewlett-Packard, Waldbron, Germany) MSD-5970 mass spectrometer directly interfaced to a HP 5890 gas chromatograph equipped with a 17 m HP crosslinked methylsilicone Ultra-1 column (ID 0.20 mm, f_{th} 0.11 μm). The oven temperature program was as follows: 120°C (0 min)–70°C/min → 181°C (0.2 min)–4°C/min → 234°C (0.1 min)–30°C/min → 300°C (3 min). The electron energy was set at 70 eV and the ion source temperature at 270°C. A total of 0.5 μl was injected splitless.

2.3.2. Endogenous steroid profile and quantitation of noretiocholanolone and norandrosterone in urine

The quantitation of norandrosterone and noretiocholanolone as well as the monitoring of the endogenous steroids was done by GC/MS in selected ion monitoring (SIM) (Table 1) on a HP MSD-5973 mass spectrometer directly interfaced to a HP 6890 gas chromatograph equipped with a 17 m HP crosslinked methylsilicone Ultra-1 column (ID 0.20 mm, f_{th} 0.11 μm). The oven temperature program was as above. The electron

energy was set at 70 eV and the ion source temperature at 270°C. A total of 0.5 μl was injected splitless. One point calibration was performed by analysing a steroid free urine sample spiked with norandrosterone, noretiocholanolone, testosterone, epitestosterone and 4-androstene-3,17-dione at 50 ng/ml.

2.4. GC/MS-MS

Quantitative analysis of low concentrations of norandrosterone and noretiocholanolone was done by tandem mass spectrometry on a Finnigan MAT GCQ (Finnigan MAT, Austin, USA) instrument operating in EI-mode. The GC-column was

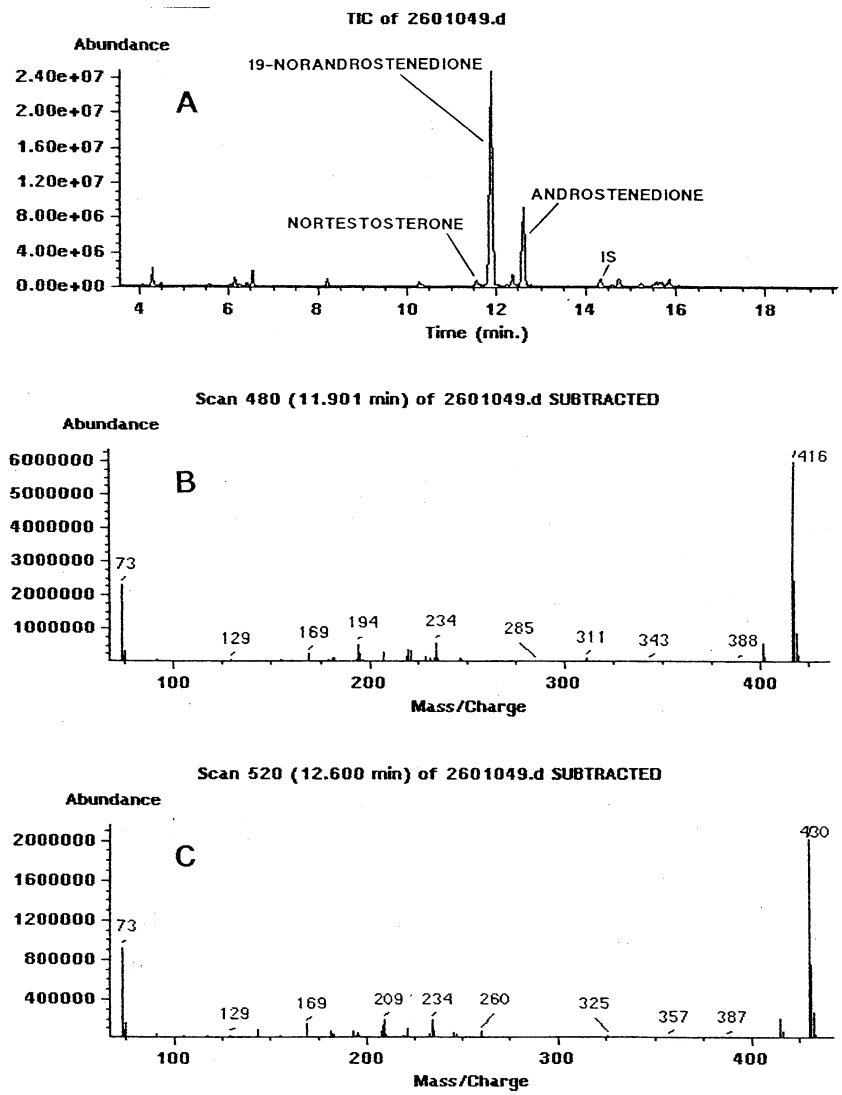

Fig. 2. (A) Total ion gas chromatrogram of the collected fraction. (B) Full scan mass spectrum of trimethylsilylated 19-nor-4-androstene-3,17-dione in the nutritional supplement. (C) Full scan mass spectrum of trimethylsilylated 4-androstene-3,17-dione in the nutritional supplement.

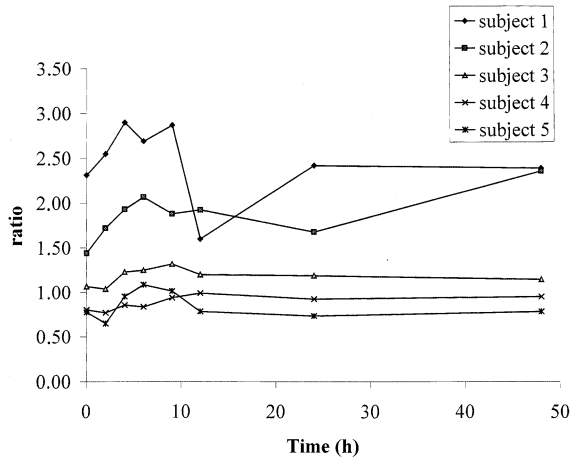

Fig. 3. Urinary testosterone/epitestosterone ratios in five male volunteers after ingestion of the contaminated food supplement.

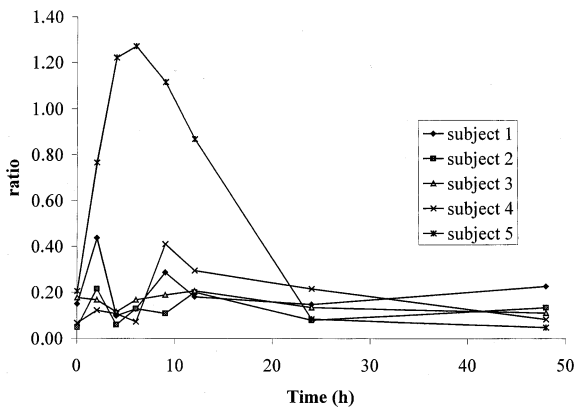

Fig. 4. Urinary 4-androstene-3,17-dione/epitestosterone ratios in five male volunteers after ingestion of the contaminated food supplement.

a 17m HP Ultra-1MS (ID 0.20 mm, f_{th} 0.33 μm) column. The oven temperature was as follows: 120°C (0.5 min)–40°C/min → 220°C (0.1 min)–4°C/min → 270°C (0.1 min)–30°C/min → 300°C (5 min). One μl was injected splitless and $m/z = 405$ was selected as parent ion. The collision energy was set at 1.1. One point calibration was performed by concurrently analysing a urine sample spiked with norandrosterone and noretiocholanolone at 2 ng/ml.

2.5. Excretion studies

One capsule of the food supplement was taken orally by five healthy male volunteers. Urine was collected before administration and quantitatively during the first 12 h, i.e. 2, 4, 6, 9, 12 h post administration. Additional urine samples were collected after 24, 48, 72, 96, 120, 144 and 168 h.

2.6. Analytical procedures

2.6.1. Analysis of food supplements

Five ml NaOH (1 M) was added to 30 mg of a food supplement (mixed sample of five capsules) in a screw capped tube. After addition of 100 μl of the internal standard (17α-methyltestosterone, 100 μg/ml MeOH) extraction was performed by rolling (1 h) with 5 ml of n-pentane, followed by centrifugation. The organic layer was separated and evaporated under OFN at 40°C. The residue was dissolved in 200 μl mobile phase (2% acetonitrile). A total of 20 μl was injected into the HPLC. The fraction between 15.2 and 18.3 min was collected and evaporated under OFN at 70°C. The residue was derivatised with 100 μl MSTFA/NH_4I/ethanethiol (380:1:2, v/w/v) for 20 min at 80°C. After derivatisation the extract was diluted 1/5 with the derivatising mixture and analysed in full scan mode.

2.6.2. Urine analysis

One ml of sodium acetate buffer (pH 5.2, 1 M) and 50 μl β-glucuronidase were added to 5.0 ml of urine. The mixture was hydrolysed for 2.5 h at 56°C. After addition of 50 μl of the internal standard (17α-methyltestosterone, 2 μg/ml MeOH) and alkalisation ($NaHCO_3/K_2CO_3$, 2:1) extraction was performed by rolling (20 min) with 5 ml n-pentane, followed by centrifugation. The organic layer was separated and evaporated under OFN. The residue was derivatised with 100 μl MSTFA/NH_4I/ethanethiol (380:1:2, v/w/v) for 20 min at 80°C. Urine samples till 96 h post administration were analysed on the HP-MSD in SIM mode. Samples taken after 96 h were analysed on the GCQ in MS-MS mode. Urine samples were diluted when appropiate.

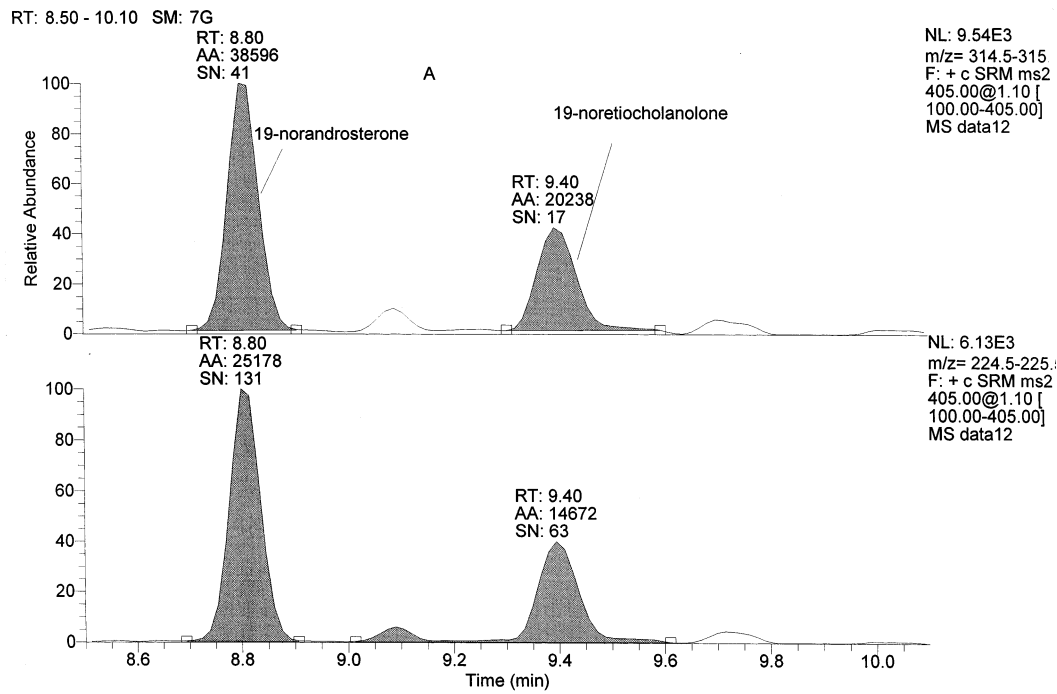

Fig. 5. (A) Ion gas chromatograms ($m/z = 315$ and $m/z = 225$) of trimethylsilylated 19-norandrosterone and 19-noretiocholanolone. (B) Subtracted MS/MS-spectrum of trimethylsilylated 19-norandrosterone. (C) Subtracted MS/MS-spectrum of trimethylsilylated 19-noretiocholanolone.

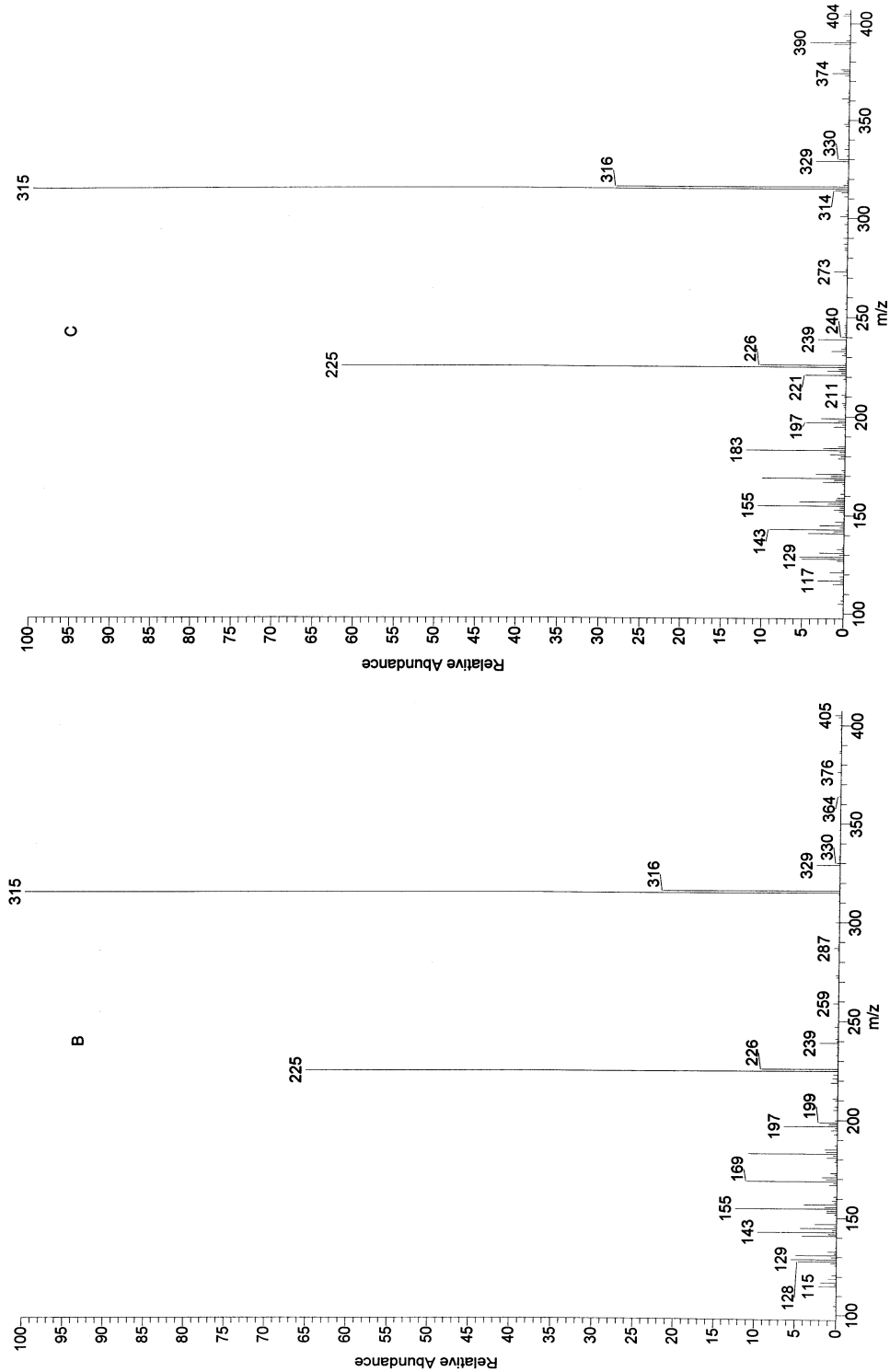

Fig. 5. (*Continued*)

3. Results and discussion

3.1. Food supplement

The sample matrix of the nutritional supplement did not allow for direct analysis by GC/MS. Therefore HPLC was used as a screening and clean-up method. The HPLC chromatograms of the standard mixture and the collected fraction (15.2–18.3 min) of the investigated food supplement are presented in Fig. 1.

The presence of 19-nor-4-androstene-3,17-dione and 4-androstene-3,17-dione (both not listed on the label) in the collected fraction was confirmed by GC/MS in full scan mode. Total ion chromatogram and mass spectra are presented in Fig. 2.

Concentrations of approximately 0.9 and 6.3 mg/g were found for respectively 4-androstene-3,17-dione and 19-nor-4-androstene-3,17-dione. Hence, one capsule of the nutritional supplement contained 0.7 mg 4-androstene-3,17-dione and 4.8 mg 19-nor-4-androstene-3,17-dione.

3.2. Excretion study

4-Androstene-3,17-dione is a precursor of testosterone in humans, therefore an increase in the urinary testosterone as well as 4-androstene-3,17-dione concentration can be expected after ingestion [5]. From the excretion studies it appeared that neither the ratio testosterone/epitestosterone nor the ratio androstenedione/epitestosterone significantly increased (Figs. 3 and 4). These ratios are both markers for the use of exogenous testosterone and androstenedione. IOC doping regulations state that a ratio testosterone/epitestosterone greater than 6 in urine is constitutes an offence, unless there is evidence that an unusually high ratio could be due to physiological or pathological conditions (e.g. low epitestosterone excretion, enzyme deficiencies) [6]. Van Eenoo et al. [5] stated that a ratio 4-androstene-3,17-dione/epitestosterone greater than 1.2 is considered as indicative for exogenous 4-androstene-3,17-dione intake. In only one urine sample a ratio 4-androstene-3,17-

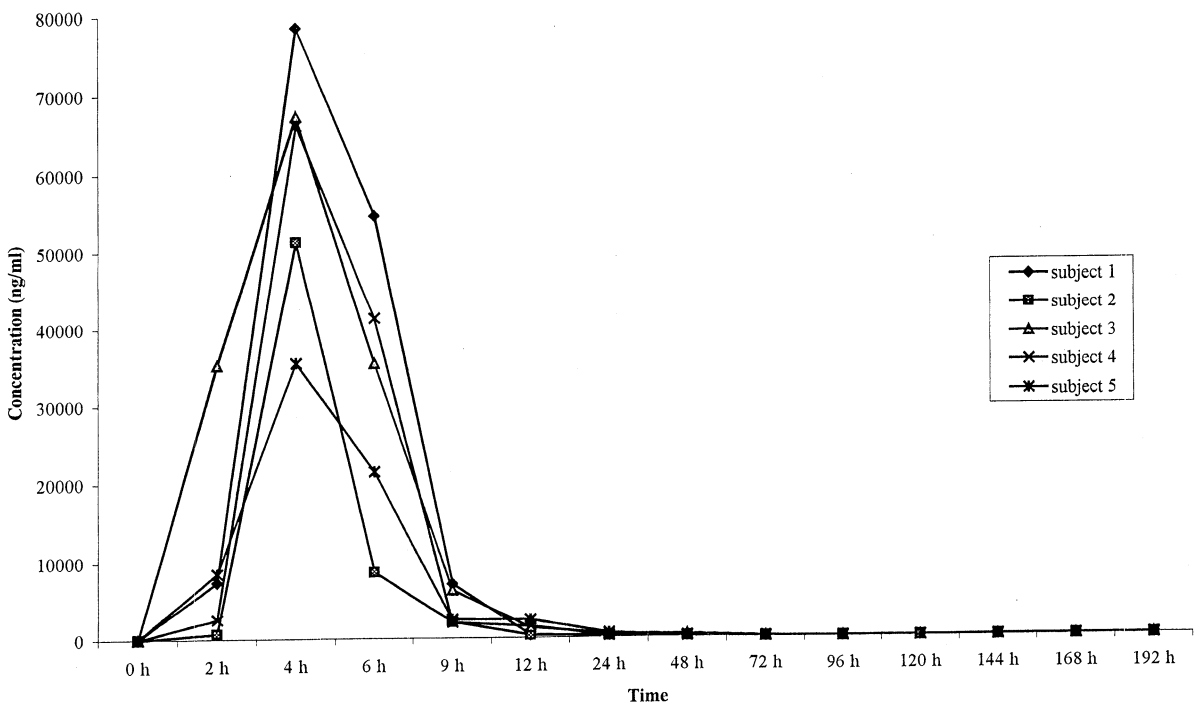

Fig. 6. Urinary concentrations of 19-norandrosterone in five male volunteers after ingestion of the contaminated food supplement.

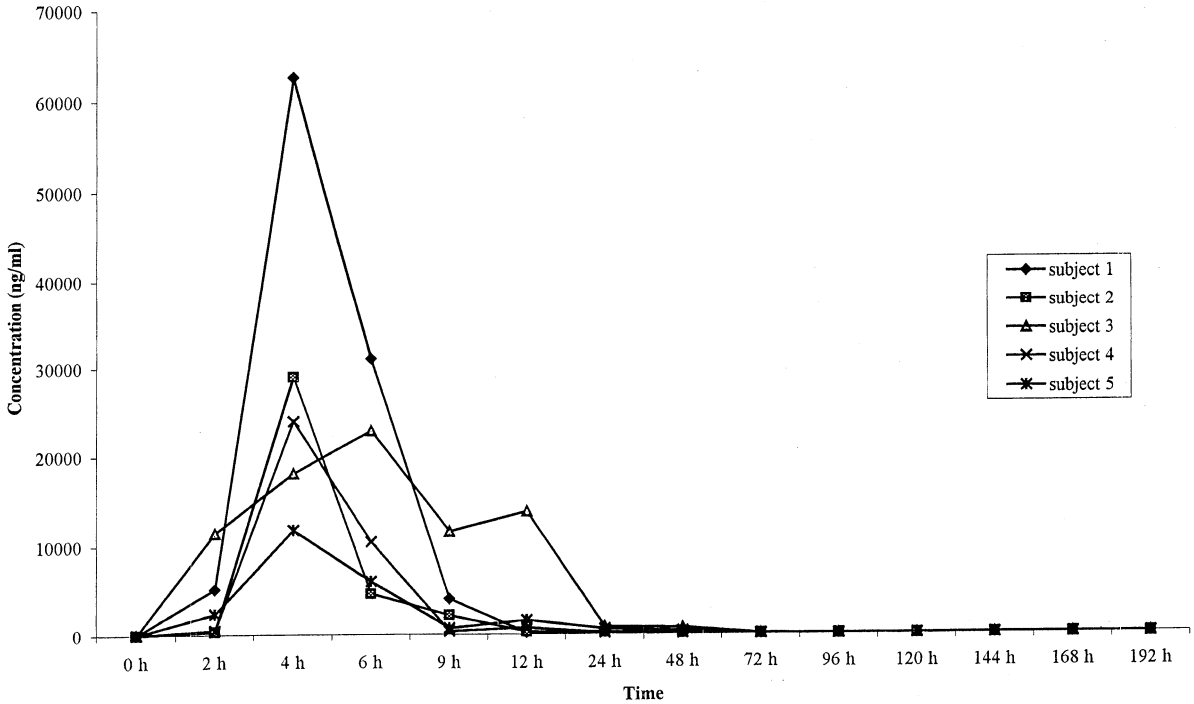

Fig. 7. Urinary concentrations of 19-noretiocholanolone in five male volunteers after ingestion of the contaminated food supplement.

dione/epitestosterone greater than 1.2 was detected. None of the volunteers were found positive according to the criteria stated by the IOC. This could be due to the low concentration of 4-androstene-3,17-dione in the supplement and the low biological availability as a result of the first pass effect.

The major urinary metabolites of nandrolone are 19-noretiocholanolone and 19-norandrosterone [4,7]. The ion chromatograms and MS/MS-spectra obtained in a 72-h sample are given in Fig. 5. The urinary excretion profiles of these substances are shown in Figs. 6 and 7. This profile indicates that 19-norandrosterone and 19-noretiocholanolone are mainly excreted in the first 24 h after oral intake.

According to the IOC doping threshold level for norandrosterone of 2 ng/ml in urine of males [4], positive findings were found until 48–144 h post administration (Fig. 8).

This study points out that there is a possibility to take unwillingly prohibited substances in the form of food supplements. Athletes should therefore consider that nutritional supplements do not pass appropriate quality tests as for registered drugs and that there is no guarantee that these supplements contain no prohibited substances. Moreover no IOC banned substances were printed on the label of the analysed supplement.

Another consideration is the daily intake of contaminated supplements. In this case the manufacturer recommended seven capsules on a daily base. One can imagine what the health effects will be after the intake of these supplements during an extended period of time. Therefore measures should be taken to inform the athletes and to stop the production of these unlicensed nutritional supplements.

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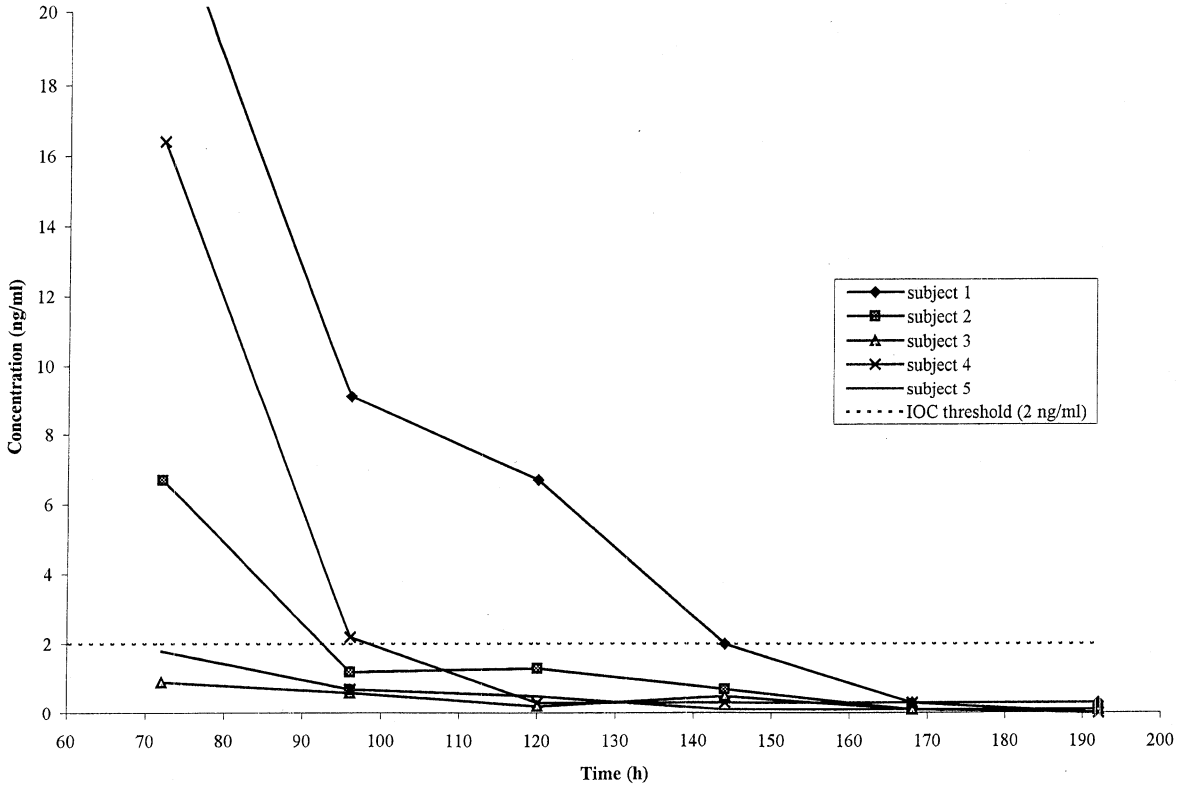

Fig. 8. Urinary concentrations of 19-norandrosterone in five male volunteers from 60 to 192 h after the ingestion of the contaminated food supplement.

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