Quantification and Profiling of 19-Norandrosterone and 19-Noretiocholanolone in Human Urine after Consumption of a Nutritional Supplement and Norsteroids*

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Abstract

Nandrolone is one of the synthetic anabolic steroids banned in sports and has been a popular substance abused by athletes in recent years. One of its major metabolites, 19-norandrosterone (19-NA), has been used as a determinant for drug violations in sports. Current reports regarding nandrolone-positive cases have been related to intake of some nandrolone-free nutritional supplements. The aim of this study was to learn whether if a nutritional supplement sold by over-the-counter (OTC) nutritional stores could yield the same metabolic products as that of nandrolone. If so, what is (are) the substance(s) that contributed to the nandrolone metabolites? To determine the content of an OTC nutritional supplement, a tablet was dissolved in methanol, followed by *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA)-trimethyliodosilane (TMIS) derivatization prior to gas chromatography–mass spectrometry (GC–MS) analysis. The collected urine samples underwent extraction, enzymatic hydrolysis, and derivatization before the analyses of GC–MS. The results showed that seven anabolic steroids were found as contaminants in the nutritional supplement, in addition to six that were listed in the ingredients by the manufacturer. We confirmed previous reports that administration of the OTC supplement could produce a positive urine test for nandrolone metabolites. Furthermore, the results from excretion studies showed that 19-NA and 19-noretiocholanolone (19-NE) were present in urine after consuming the nutritional supplement, nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol. The 19-NA concentrations in urine were generally higher than that of 19-NE (19-NA/19-NE ratio > 1.0) especially during the early stage of excretion, that is, before 6 h post-administration. After this period of time, the concentrations of 19-NA and 19-NE fluctuated and might even have reversed (19-NA/19-NE ratio < 1.0) in their ratio, that is, higher yield in 19-NE than that in 19-NA. On the basis of this study, we postulate that some doping violations of nandrolone could be attributed by indiscriminate administration of the OTC nutritional supplements that contained 19-norsteroids.

Introduction

The use of nandrolone (19-nortestosterone) by athletes became popular in the late 1950s (1). Nandrolone is the most frequently abused substance in doping control in the past decade because it does not convert to estrogens and does not have androgenic side effects (2). Two major urinary metabolites, 19-norandrosterone (19-NA) and 19-noretiocolanolone (19-NE), have been used as determinants for doping of nandrolone. In 1998, the IOC advised the accredited doping control laboratories to use a 19-NA cutoff value of 2 ng/mL for male athletes and 5 ng/mL for female athletes to avoid false positives as a result of consumption of meat from nandrolone-treated animals or endogenous production (3–8). Nevertheless, the possibility of false-positive doping tests was raised as a result of the endogenous metabolic complicity occurred on the 19-NA and 19-NE metabolites (9).

Nandrolone has been identified in human ovarian follicular fluid as a possible intermediate in the multistep enzymatic conversion of androgen to estrogen (10). During the course of pregnancy, 19-NA showed an increase in urine (11), and human chorionic gonadotropin (hCG) administration also stimulated 19-NA excretion in urine (12). Exercise has been shown to affect the urinary excretion of nandrolone metabolites (13,14). Furthermore, 19-NA was found to be the main urinary metabolite of norandrostenedione and norandrostenediol and a minor metabolite of oral contraceptives such as norethisterone (15). Oral administration of nandrolone and other 19-norsteroids was primarily excreted to 19-NA, 19-NE, and norepiandrosterone, the latter found exclusively as its sulfoconjugate while the first two are predominantly excreted as glucuronide derivatives (16). In the metabolic pathway, the concentrations of 19-

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NA and 19-NE were found with ratio of 72:28 (17).

The prevalence of nandrolone abuse was evident; internationally, 212 cases were found in 1995 and 232 cases were found in 1996 (3). From 1998 to 2001, nandrolone-positive findings reported by the IOC-accredited laboratories ranged from 0.17% to 0.65% (16). In our laboratory, between 1998 and 2001, nandrolone was ranked the highest (44%), followed by metandienone (31%) and stanozolol (19%) among anabolic steroids offenses (18).

Recent studies have demonstrated that the administration of over-the-counter (OTC) nutritional supplements containing prohormones of nandrolone, including 19-nor-4-androstene-3,17-dione, 19-nor-4-androstene-3β,17β-diol, and 19-nor-5-androstene-3β,17β-diol, could also result in positive urine test results for the nandrolone metabolite, 19-NA (19–22).

In this study, we analyzed the contents of an OTC nutritional supplement and performed a series of excretion studies, first to evaluate whether the nutritional supplement will metabolize to 19-NA and 19-NE and second to identify the sources that contribute to 19-NA and 19-NE in urine if the supplement yields the nandrolone metabolites.

Experimental

Nutritional supplement

The nutritional supplement Androstat6 was purchased from BODYONICS, USA. Anabolic steroids labeled as ingredients are 4-androstene-3,17-diol (4-androstenediol, 125 mg); 4-androstene-3,17-dione (4-androstenedione, 5 mg); 5-androstene-3,17-dione (5-androstenedione, 5 mg); 5-androstene-3,17-diol (5-androstenediol, 5 mg); 19-nor-4-androstene-3,17-dione (19-nor-4-androstenedione, 5 mg); and 19-nor-5-androstene-3,17-dione (19-nor-5-androstendiol, 5 mg).

Chemicals, solvents, and reagents

Acetic acid, anhydrous dibasic sodium phosphate, monoacid sodium phosphate, potassium carbonate, sodium acetate, sodium bicarbonate, acetonitrile, dichloromethane, diethyl ether, hexane, methanol, ethyl acetate, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), trimethyliodosilane (TMIS), and β-glucuronidase from *Escherichia (E.) coli* were purchased from Sigma (St. Louis, MO). Ethanethiol was obtained from Fluka (Milwaukee, WI). All chemicals and solvents were of analytical grade or better. Deionized water was generated with a Millipore Milli-RO4/Milli Q water purification system.

Reference anabolic steroids

19-Norandrosterone (3α-hydroxy-5α-estran-17-one; 19-NA) and 19-noretiocholanolone (3α-hydroxy-5β-estran-17-one; 19-NE) were obtained from Cerilliant (Austin, TX). Nandrolone (17β-hydroxyestr-4-en-3-one), 5β-androstane-3α,17β-diol, 19-nor-4-androsten-3,17-dione (norandrostenedione), 5-androsten-3β,17β-diol (5-androstenediol), 5α-androstane-3β, 17β-diol, and testosterone were purchased from Sigma. 5-Androsten-3β-ol-17-one (dehydroepiandrosterone; DHEA), 4-androsten-3β,17β-diol (4-androstenediol), 4-androsten-3,17-dione (4-androstenedione), 5-androsten-3,17-dione (5-androstenedione), and methylandrostanediol (17α-methyl-5α-androstan-3β,17β-diol; internal standard) were purchased from Steraloids (Wilton, NH).

Urinary samples

Urine samples were obtained from the volunteers after administering the following substances: the nutritional supplement, nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol. Urine samples were collected from the volunteers before and after the supplement was administered. Specifically, urine specimens were collected before administration of each substance and at 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, and 168 h post-administration. Urine that was collected in each time period was mixed and stored at –20°C before the analysis. Urinary pH and specific gravity of the samples were measured and recorded. The excretion studies were approved by the Human Subjects Research Review Committee.

Derivatization mixture

A one-step derivatization process was used to convert steroids to the trimethylsilyl (TMS) enol–TMS ether derivatives (23,24). Trimethyliodosilane solution (TMIS; 0.1M) was first prepared by mixing trimethyliodosilane, triethylamine, and dichloromethane (70:1:430). The final TMS derivatization mixture was then prepared by adding 3.0 mL of MSTFA, 60 µL of ethanethiol, and 60 µL (0.1M) of trimethyliodosilane solution (TMIS). The mixture was then stored at –20°C.

Nutritional supplement sample preparation

After pulverization with a grinder, one tablet of the nutritional supplement was dissolved in 5 mL deionized water. The sample vials including controls, water, and the supplement were prepared by adding 30 µL of sample. The samples were evaporated to dryness under nitrogen air stream. An aliquot of 50 µL derivatization mixture (as described) was added and heated at 70°C for 30 min. One microliter of the derivatized sample was injected into the gas chromatograph–mass spectrometer (GC–MS).

Urine specimens collected from the excretion study

The urine specimens were collected from three healthy, male volunteers who were administered one dose (two tablets) of the nutritional supplement, nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol. The urine specimens were pretreated according to the procedure described here.

Solid-phase extraction (SPE)

A Sep-Pak Plus C_{18} cartridge (Waters) was first conditioned with 5 mL (2×) of methanol and 5 mL (2×) of deionized water. With a volume of 1.5 to 10 mL of urine, depending upon its specific gravity, were mixed an equal volume of acetate buffer and 25 µL internal standard in a 20-mL glass tube. After passing through the SPE cartridge, the urine sample was washed with water (5 mL) and hexane (3 mL). The sample was then collected in a 15-mL glass tube by rinsing the cartridge with methanol

(5 mL). The methanolic extract was evaporated to dryness at 40°C under nitrogen.

Enzymatic hydrolysis

Investigations of the metabolism of anabolic androgenic steroids have shown that most of the conjugated steroids and their main metabolites can be hydrolyzed with β-glucuronidase from *E. coli* (19). To the tube, 1 mL of phosphate buffer (pH 6.9) and 50 mL of β-glucuronidase from *E. coli* in phosphate buffer were added. The enzymatic hydrolysis was carried out at 50°C for 60 min.

Liquid–liquid extraction

Following enzymatic hydrolysis step, approximately 100 mg of a $NaHCO_3$ and Na_2CO_3 mixture (10:1, w/w; pH 9.0) were added to the glass tube. In order to isolate the deconjugated and unconjugated anabolic steroids from the sample, a liquid–liquid extraction was performed by the addition of 5 mL diethyl ether followed by vigorous agitation and centrifugation (2000 rpm; 8 min). The organic layer was transferred and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 200 µL methanol, which was then transferred to a conical injection vial.

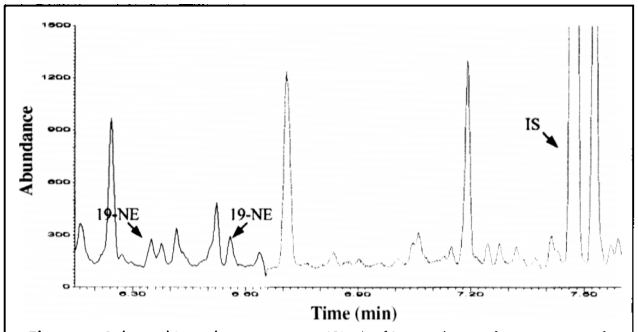

Figure 1. Selected ion chromatogram (SIM) of ion *m/z* 405 for 19-NA and 19-NE at lowest detectable concentration (0.5 ng/mL) in urine. 19-NA (RT = 6.37 min); 19-NE (RT = 6.58 min); and internal standard (methylandrostanediol; RT = 7.47 min).

Table I. Recovery of 19-NA and 19-NE in Urine

Amount (ng/mL)	19-NA (%Recovery ± %CV)	19-NE (%Recovery ± %CV)
4	110.4 ± 6.8	131.9 ± 6.2
10	110.7 ± 1.5	129.7 ± 4.5
50	85.8 ± 4.4	88.2 ± 4.9

Table II. Accuracy and Precision for the Determination of 19-NA and 19-NE in Urine

Amount (ng/mL)	Intra-assay (n = 5)		Interassay (n = 4)	
	%Target	%CV	%Target	%CV
19-NA				
6	104	2.0	108	2.2
20	106	4.1	107	2.7
100	103	1.2	105	1.7
19-NE				
6	100	2.3	108	6.4
20	100	4.7	107	1.3
100	101	1.8	105	1.3

Derivatization

The solvent in the injection vial was evaporated to dryness under nitrogen. Subsequently an aliquot of 50 µL of TMS derivatization mixture was added and heated at 70°C for 30 min. A 1-µL sample was injected into the GC–MS.

Instrumentation and conditions

A Hewlett-Packard HP 6890 GC coupled with a 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA) was used for the analyses. An HP-5MS cross-linked 5% diphenyl and 95% dimethylpolysiloxane capillary column (25 m × 0.25-mm i.d., 0.33-µm film thickness) was used for GC separation. Helium was used as the carrier gas. The temperature of the GC injection port was maintained at 270°C, and the transfer line temperature was 300°C. The MS was tuned with perflurotributylamine (PFTBA) to optimize the selected ions of *m/z* 69, 219, and 502. The GC temperature program was operated as follows: Initial temperature was 100°C and held for 1 min, increased by 20°C/min to 220°C, then 4.4°C/min to 320°C, and held for 5.27 min.

The MS was operated in the electron impact (EI) mode at electron ionization energy of 70 eV. Selected ion monitoring (SIM) with three target ions was used for screening of individual steroid tested in this study and full scan mode was followed for confirmation. The mass spectrum was obtained by scanning from *m/z* 50 to 550.

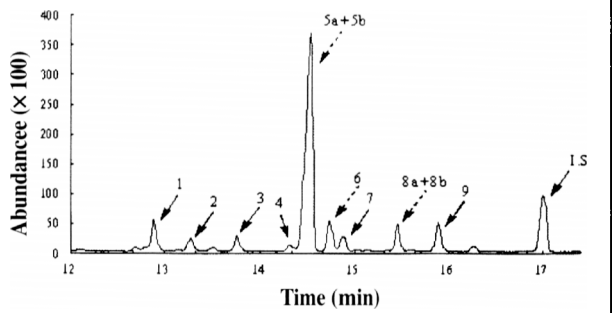

Figure 2. Ion chromatograms of the nutritional supplement analyzed by GC–MS. Each number next to the arrow indicates one anabolic steroid. The dashed arrows represent the anabolic steroids that were labeled on the supplement ingredients list and also detected by GC–MS, whereas the solid arrows represent the steroids not labeled by the manufacturers but detected by GC–MS. Anabolic steroids peak identification is as follows: 1, undetermined; 2, 19-nor-5-androsten-3β,17β-diol bis-TMS; 3, 19-nor-4-androsten-3β,17β-diol bis-TMS; 4, 5-androsten-3β-ol-17-one bis-TMS; 5a, 19-nor-4-androsten-3,17-dione bis-TMS and 5b, 4-androsten-3β,17β-diol bis-TMS; 6, 5-androsten-3β,17β-diol bis-TMS; 7, 5α-androstane-3β,17β-diol bis-TMS; 8a, 4-androsten-3,17-dione bis-TMS and 8b, 5-androsten-3,17-dione bis-TMS; and 9, testosterone bis-TMS. Note: peaks on 5a and 5b were co-eluted, whereas 8a and 8b were two indistinguishable compounds in terms of their RRTs and spectra.

Calibration curves

Quantification of anabolic steroids in nutritional supplement. One tablet (n = 3) of the nutritional supplement was thoroughly dissolved in 50 mL methanol. The calibration solutions were prepared in triplicates by adding appropriate quantities of authentic standards to methanol. In order to avoid coelution of some compounds, two sets of samples were prepared and analyzed separately. Each set included following concentrations of the reference standards: 2.5, 5, 10, 20, 40, 50, and 80 µg/mL. In one set, five compounds were included: 19-nor-5-androsten-3β,17β-diol, 5-androsten-3β-ol-17-one, 4-androsten-3β,17β-diol, 5-androsten-3β,17β-diol, and 4-androsten-3,17-dione. In the other set, 19-nor-4-androsten-3β,17β-diol, 19-nor-4-androsten-3,17-dione, 5α-androstane-3β,17β-diol, 5-androsten-3,17-dione, and testosterone were included. The internal standard (methylandrostanediol, 10 µg/mL) was added in each sample. One target ion from each of the aforementioned steroids was quantified by GC–MS. The calibration curve was constructed by plotting the peak-area ratios of the standards and the internal standard versus the concentrations.

Table III. Anabolic Steroids Labeled in the Ingredients of the Nutritional Supplement (Androstst6) and Those Detected by GC–MS

	Anabolic Steroid	RT (min)	RRT*	Target Ions	Labeled (mg)	Detected (mg†)
1	Undetermined	12.90	0.75	239,405,419,434		
2	19-Nor-5-androsten-3β, 17β-diol bis-TMS	13.30	0.78	420,405,240,330‡	–§	1.3#
3	19-Nor-4-androsten-3β, 17β-diol bis-TMS	13.54	0.79	405,330,240,420‡	–	0.7#
4	5-Androsten-3β-ol-17-one bis-TMS	14.34	0.84	327,417,432‡	–	31.9#
5a	19-Nor-4-androsten-3, 17-dione bis-TMS	14.58	0.84	194,401,416‡	5	3.1
5b	4-Androsten-3β, 17β-diol bis-TMS	14.58	0.85	239,405,419,434‡	–	28.5#
6	5-Androsten-3β, 17β-diol bis-TMS	14.79	0.86	344,434,239‡	–	2.6#
7	5α-Androstane-3β, 17β-diol bis-TMS	14.93	0.87	346,436,421‡	–	1.2#
8a	4-Androsten-3, 17-dione bis-TMS	15.54	0.91	234,415,430‡	5	13.3
8b	5-Androsten-3, 17-dione bis-TMS	15.54	0.91	234,415,430‡	5	25.5
9	Testosterone bis-TMS	15.97	0.93	417,432‡	–	2.6#
10	19-Nor-5-androsten-3, 17-diol**				5	
11	4-Androsten-3,17-diol**				125	
12	5-Androsten-3,17-diol**				5	
13	Methylandrostanediol (internal standard)	17.10		143,435,450‡		

* RRT indicates the relative retention time and is derived from the ratio of RT of analyte/RT of internal standard.
† Mean value (n = 3) of amount detected in the nutritional supplement.
‡ Indicates molecular ion.
§ Indicates the anabolic steroid unlabeled in the nutritional supplement by the manufacturer.
# Indicates the anabolic steroid was not labeled in the nutritional supplement by the manufacturer but detected by GC–MS.
** Indicates reference standard was not available.

Quantification of nandrolone metabolites. Two separate concentration levels were used to construct calibration curves for both 19-NA and 19-NE. The low concentration level contained six concentrations of 19-NA and 19-NE, 0.5, 1, 1.5, 2, 3, 4, and 6 ng/mL, whereas the high concentration level included 10, 20, 40, 100, 200, and 400 ng/mL. A batch of child's urine, used for constructing the calibration curves, was prepared by passing through Sep-Pak Plus C_{18} cartridge to ensure it was drug free. The standards in triplicates were spiked with the known concentrations of 19-NA and 19-NE. Quantification was carried out by SIM mode with a selected ion m/z 405 for 19-NA and 19-NE and an ion m/z 435 for the internal standard. The injection volume of the samples was 2 µL. The average peak-area ratio of the standard, determined by the ratio of a selected ion of the standard to the selected ion of the internal standard, was used to construct a calibration curve. The slope obtained from the calibration curve for the standard was used to quantify the concentration of 19-NA and 19-NE in the sample. Although correction of concentrations by creatinine was shown to give better results than that by specific gravity, Donike et al. (25) claimed that if only the specific gravity is available for correction, a specific gravity between 1.007 and 1.020 g/mL should only be used. Moreover, samples with a specific gravity lower than 1.007 should be excluded and samples with a specific gravity higher than 1.020 g/mL not corrected. The final concentrations of 19-NA and 19-NE in urine samples were, therefore, further corrected on the basis of the formula developed by Donike et al. (25).

$$Ccorr = \frac{(1.020 - 0.998) \times Cm}{d - 0.998} \qquad \text{Eq. 1}$$

[$Ccorr$ = corrected concentration; d = measured specific gravity; Cm = measured concentration; 0.998 = specific gravity of water]

Results

Limit of detection (LOD), limit of quantitation (LOQ), and calibration curve

The LOD is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence to indicate that the analyte concentration is greater than zero. LOD is determined from analysis of a sample in a matrix containing the analyte (26). Accordingly, a single diagnostic ion (m/z 405) was used to determine the LOD of 19-NA and 19-NE by GC–MS. The LODs of 19-NA and 19-NE were evaluated to be 0.16 ng/mL and 0.06 ng/mL, respectively. The LOQs of 19-NA and 19-NE were 0.5 ng/mL and 0.2 ng/mL, respectively, as determined by multiplying the LODs of 19-NA (0.16 ng/mL) and 19-NE (0.06 ng/mL) by a factor of 3. Figure 1 shows 19-NA and 19-

NE total ion chromatogram of selected ions (*m/z* 420, 405, and 315) when 0.5 ng/mL of the listed standards were spiked in urine and analyzed by GC–MS.

Two (low and high) concentration levels of calibration curves for 19-NA and 19-NE were constructed. For the low concentration level calibration curve, the assay was linear from 0.5 to 6 ng/mL with $y = 0.0144 - 0.0001$ ($r^2 = 0.9953$) for 19-NA and $y = 0.0152 + 0.0026$ ($r^2 = 0.9989$) for 19-NE. For the high concentration calibration curve, the assay was linear from 10 to 400 ng/mL with $y = 0.00117 - 0.0859$ ($r^2 = 0.9984$) for 19-NA and $y = 0.0095 + 0.1068$ ($r^2 = 0.9989$) for 19-NE.

Recovery, accuracy, and precision

To determine extraction efficiency of the sample preparation procedure, three target concentrations of 19-NA and 19-NE were employed and 5 replicates for each concentration were analyzed. The recovery of the target concentrations at 4, 10, and 50 ng/mL was 110.4%, 110.7%, and 85.7%, respectively, for 19-NA and with 131.9%, 129.7%, and 88.2%, respectively, for 19-NE (Table I).

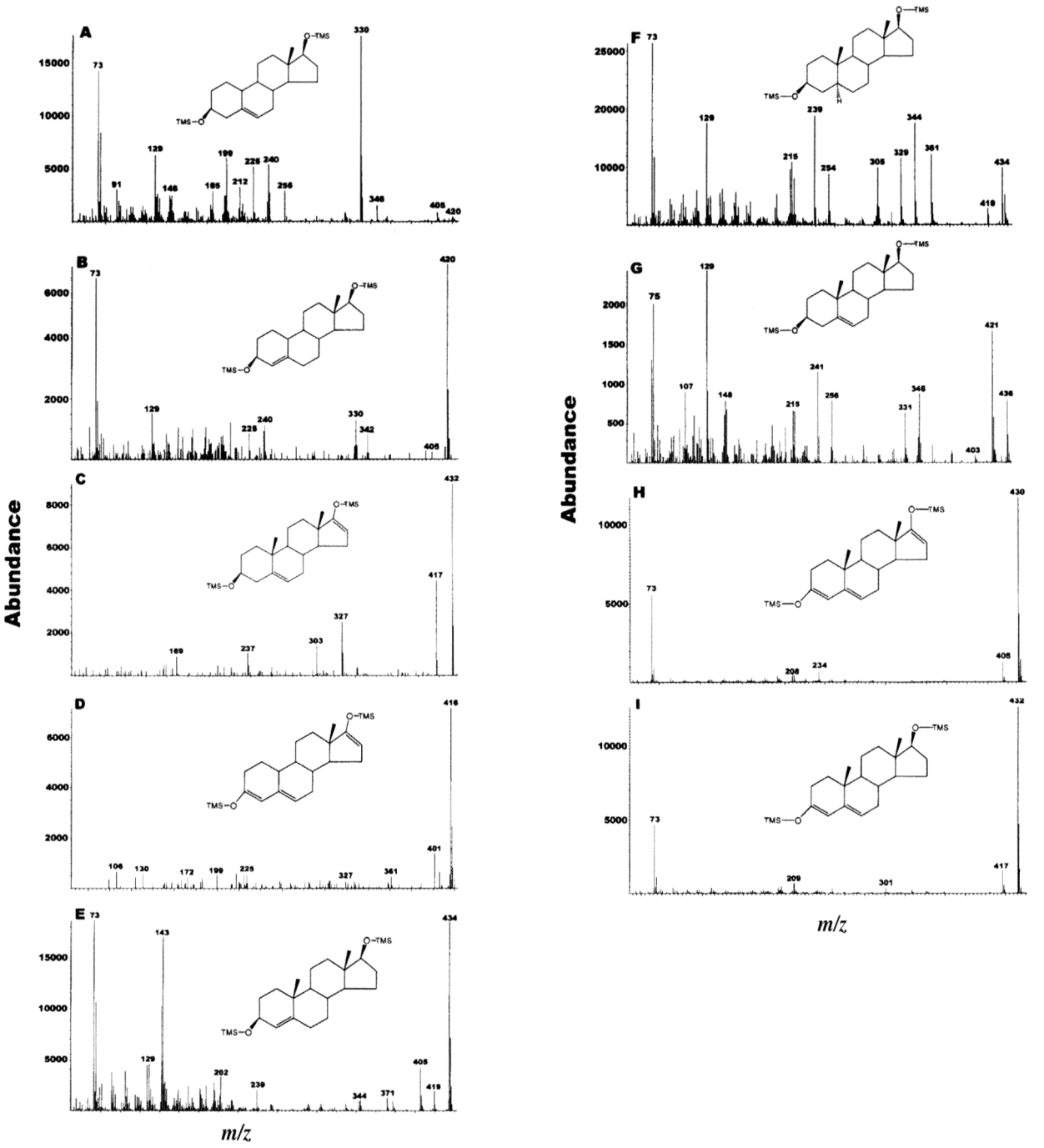

Figure 3. EI mass spectra of anabolic steroids detected in the nutritional supplement: 19-nor-5-androsten-3β,17β-diol bis-TMS (A); 19-nor-4-androsten-3β,17β-diol bis-TMS (B); 5-androsten-3β-ol-17-one bis-TMS (C); 19-nor-4-androsten-3,17-dione bis-TMS (D); 4-androsten-3β,17β-diol bis-TMS (E); 5α-androstane-3β,17β-diol bis-TMS (F); 5-androsten-3β,17β-diol bis-TMS (G); 4-androsten-3,17-dione bis-TMS and 5-androsten-3,17-dione bis-TMS (H); and testosterone bis-TMS (I).

The intra-assay accuracy and precision of 19-NA and 19-NE (n = 5) were determined from the analyses of 6 replicates at target concentrations of 6, 20, and 100 ng/mL within a single analytical batch. For the four target concentrations, the accuracy of 19-NA ranged from 103% to 106% with precision (%CV) ranging from 1.2 to 4.1; the accuracy of 19-NE ranged from 100% to 101% with precision from 1.8% to 4.7% (Table II). The interassay accuracy and precision (n = 4) were determined from 6 separate analytical runs, using the same concentrations as were used in the intra-assay studies. The interassay accuracy measured 19-NA concentrations ranged from 105% to 108% with precision ranging from 1.7% to 2.7%. The interassay accuracy measured 19-NE concentrations ranged from 105% to 108% with precision ranging from 1.3% to 6.4%.

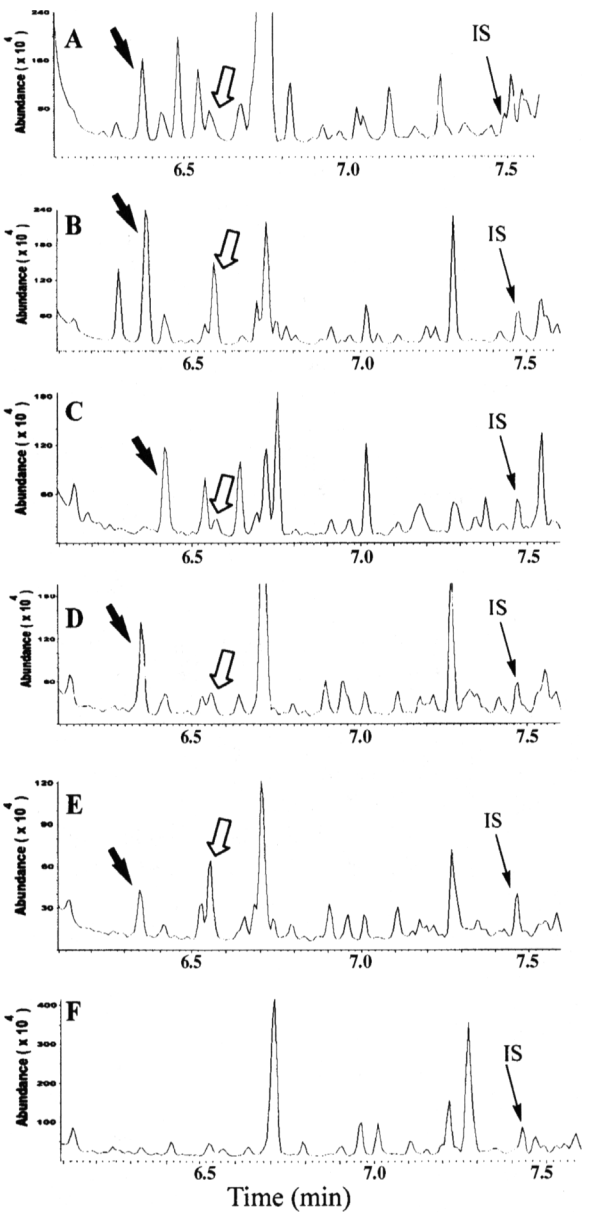

Figure 4. Representative GC chromatograms from urine of the volunteers who administered one of the following substances: nutritional supplement (A); nandrolone (B); 19-nor-4-androsten-3,17-dione (C); 19-nor-5-androsten-3β,17β-diol (D); 19-nor-4-androsten-3β,17β-diol (E); and control blank urine (F). Filled arrow = 19-NA; open arrow = 19-NE; and IS = internal standard.

Analysis of anabolic steroids in the OTC nutritional supplement

One tablet of the OTC nutritional supplement (n = 3) was dissolved in methanol and analyzed by GC–MS to determine its anabolic steroid composition. As shown in Figure 2, a total of 9 chromatographic peaks representing 10 anabolic steroids were identified. The retention times (RTs), relative retention times (RRTs), and target ions from the labeled and unlabeled (or contaminated) anabolic steroids are shown in Table III. Electron ionization (EI) mass spectra of these compounds are shown in Figure 3.

Manufacturer-labeled anabolic steroids

There were six anabolic steroids listed as ingredients for the nutritional supplement. Three of these were detected and confirmed with reference standards, including 19-nor-4-androsten-3,17-dione, 4-androsten-3,17-dione, and 5-androsten-3,17-dione. The former appeared in the chromatogram at 14.58 min (RRT = 0.84); the last two were co-eluted (RT = 15.54 min; RRT = 0.91) (Table III; Figure 2, peak 8a + 8b). Mass spectra of these anabolic steroids are shown in Figure 3D for 19-nor-4-androsten-3,17-dione and Figure 3H for 4-androsten-3,17-dione and 5-androsten-3,17-dione. The other three listed anabolic steroids, 5-androsten-3,17-diol, 4-androsten-3,17-diol, and 19-nor-5-androsten-3,17-diol, were not analyzed because of a lack of reference standards (Table III).

Unlabeled anabolic steroids

In addition to the six labeled steroids, seven unlabeled anabolic steroids were detected in the nutritional supplement (Table III) including 19-nor-5-androsten-3β,17β-diol (RT = 13.30 min; RRT = 0.78; Figure 3A), 19-nor-4-androsten-3β,17β-diol (RT = 13.54 min; RRT = 0.79; Figure 3B), 5-androsten-3β-ol-17-one (RT = 14.34 min; RRT = 0.84; Figure 3C), 4-androsten-3β,17β-diol (RT = 14.58 min; RRT = 0.85; Figure 3E), 5α-androstane-3β,17β-diol (RT = 14.93 min; RRT = 0.87; Figure 3F), 5-androsten-3β,17β-diol (RT = 14.79 min; RRT = 0.86; Figure 3G), and testosterone (RT = 15.97 min; RRT = 0.93; Figure 3I).

Amount of anabolic steroids present in the nutritional supplement

Three tablets were dissolved in methanol and quantified separately for the amount of each individual anabolic steroid contained in the supplement. The averaged amount (n = 3) of each substance contained in one tablet is presented in Table III. The amount listed for each of the three labeled anabolic steroids, 19-nor-4-androsten-3,17-dione, 4-androsten-3,17-dione, and 5-androsten-3,17-dione, was 5 mg per tablet. In contrast, the amounts detected for 19-nor-4-androsten-3,17-dione, 4-androsten-3,17-dione, and 5-androsten-3,17-dione were found to be 3.1 mg, 13.3 mg, and 25.5 mg, respectively.

Oral administration of the nutritional supplement and 19-norsteroids

When the volunteers (n = 3) orally administered one dose (two tablets) of the nutritional supplement, two primary

metabolites, 19-NA (RT = 6.37 min; RRT = 0.85) and 19-NE (RT = 6.58 min; RRT = 0.88), were identified in the chromatogram (Figure 4A). As a control, three volunteers also orally ingested 10 mg each of authentic nandrolone. The chromatographic peaks of nandrolone metabolites, 19-NA and 19-NE, from one of the volunteers appeared at 6.36 min (RRT = 0.85) and 6.57 min (RRT = 0.88), respectively (Figure 4B). To identify the sources that yielded 19-NA and 19-NE metabolites in the urine of the volunteers who consumed the supplement, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol were chosen for the excretion studies. The chromatographic results from these 19-norsteroids were comparable with those obtained from the nandrolone-administered urine. The 19-NA and 19-NE metabolites yielded from these three substances all appeared at RRT = 0.85 and 0.88, respectively (Figure 4C–4E).

Excretion profiles of 19-NA and 19-NE

The time-concentration profiles of 19-NA and 19-NE from the excreted urine samples were determined. The concentrations of 19-NA and 19-NE metabolites in urine for nutritional supplement, nandrolone, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol reached peaks as early as 2 to 6 h and declined thereafter until approximately 72 to 96 h post-administration (Figure 5).

Ratio of 19-NA and 19-NE concentrations in urine

The ratio of 19-NA and 19-NE in urine at each time point during the excretion study was also evaluated for all the substances administered (Figure 6). The ratios of 19-NA and 19-NE for the three volunteers who ingested one of each of the five mentioned substances were found to range from 1.1 to 2.4, 0.8 to 2.9, and 0.3 to 2.6, respectively, for the nutritional supplement; from 0.2 to 2.2, 0.1 to 4.5, and 0.2 to 4.0 for nandrolone; from 0.7 to 2.0, 1.8 to 8.7 and 0.3 to 3.5 for 19-nor-4-androsten-3,17-dione; from 0.3 to 3.7, 3.9 to 5.7 and 2.0 to 3.4 for 19-nor-5-androsten-3β,17β-diol; and from 0.7 to 2.2, 0.2 to 3.7 and 0.7 to 2.2, 0.2 to 3.7 and 1.4 to 4.4 for 19-nor-5-androsten-3β,17β-diol.

Cumulative amounts of 19-NA and 19-NE in urine

The cumulative amounts of 19-NA and 19-NE metabolites excreted in urine were evaluated for each of the substances consumed. Their profiles are presented in Figure 7. To obtain the cumulative amount of 19-NA and 19-NE, the amount of the metabolites at each time point was first calculated by multiplying the metabolite concentration (in ng/mL) detected by the total volume of urine (in mL) voided during that time period. The cumulative amount of each metabolite was then evaluated by adding the detected amount of the metabolite obtained with the previous amount obtained in the urine. As shown in Figure 7, the cumulative amounts of 19-NA (and 19-NE) of the three volunteers for the nutritional supplement were 932 μg (510 μg), 602 μg (288 μg), and 445 μg (394 μg); for nandrolone were 1048 μg (589 μg), 1636 μg (967 μg), and 437 μg (1138 μg); for 19-nor-4-androsten-3,17-dione were 870 μg (284 μg), 670 (428 μg), and 1138 μg (457 μg); for 19-nor-5-androsten-3β,17β-diol were 1220 μg (1678 μg), 1123 (371 μg), and 873 μg (490 μg); and for 19-nor-4-androsten-3β,17β-diol were 680 μg (156 μg), 705 μg (706 μg), and 362 μg (231 μg).

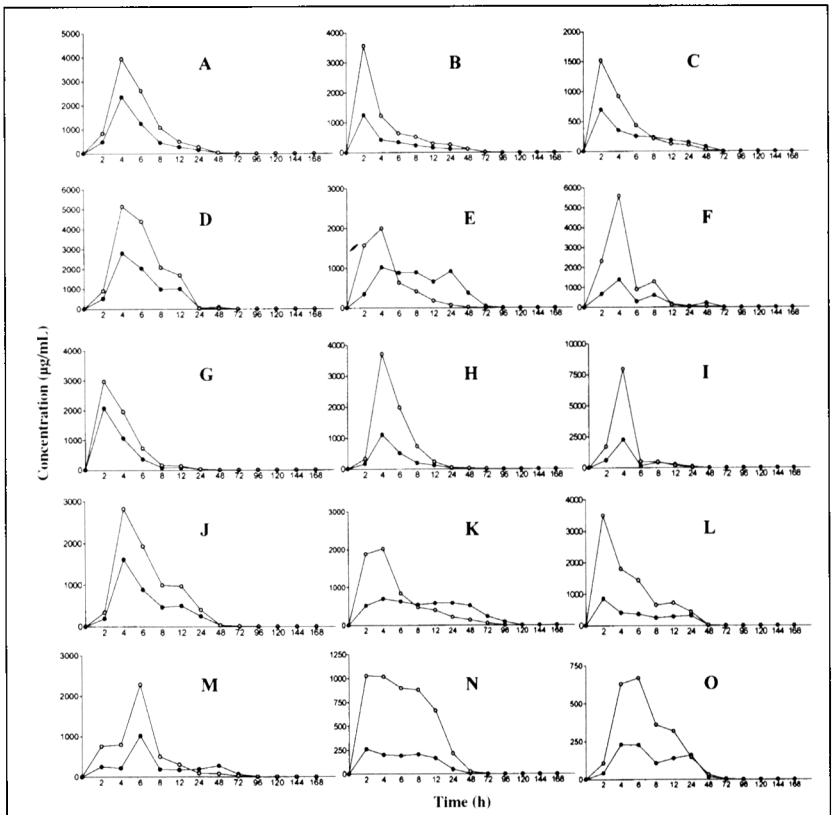

Figure 5. Urinary time-concentration profiles of 19-NA (○) and 19-NE (●) from each volunteer who orally administered various substances. Nutritional supplement (A–C); nandrolone (D–F); 19-nor-4-androstene-3,17-dione (G–I); 19-nor-5-androstene-3β,17β-diol (J–L); and 19-nor-4-androstene-3β,17β-diol (M–O).

Ratio of 19-NA and 19-NE in cumulative amounts of the substances administered

The ratios of 19-NA and 19-NE in the cumulative amounts for the three volunteers of each substance were found to be 1.1, 1.8, and 2.1 for the supplement; 0.4, 1.7, and 1.8 for nandrolone; 1.6, 2.5, and 3.0 for 19-nor-4-androstendione-3,17-dione; 1.0, 1.6, and 4.4 for 19-nor-5-androstene-3β,17β-diol; and 0.7, 1.8, and 3.0 for 19-nor-4-androstene-3β,17β-diol (Figure 8).

Discussion

Recent studies have shown that 19-NA is also the main urinary metabolite produced by the administration of 19-norsteroids (15) and in the urine of pregnant women (11). Other studies have reported that the administration of OTC nutritional supplements containing 19-norandrostenedione could also result in positive urine test results for the nandrolone metabolite, 19-NA (19–22).

In the nutritional supplement we studied, six anabolic steroids were labeled as ingredients. Three of the six-labeled anabolic steroids were detected and confirmed, including 19-nor-4-androsten-3,17-dione, 4-androsten-3,17-dione, and 5-androsten-3,17-dione; whereas 4-androsten-3,17-diol, 5-androsten-3,17-diol, and 19-nor-5-androsten-3,17-diol were not confirmed because the reference standards of these three substances were unavailable. However, of these three unconfirmed compounds, 5-androsten-3,17-diol was suspected to be 5-androsten-3β,17β-diol, and 4-androsten-3,17-diol was suspected to be 4-androsten-3β,17β-diol, as determined by their RT and mass spectra when compared to the reference standards.

Unexpectedly, we found seven unlabeled anabolic steroids contaminated in the supplement, including 5-androsten-3β,17β-diol, 4-androsten-3β,17β-diol, 19-nor-4-androsten-3β,17β-diol, 19-nor-5-androsten-3β,17β-diol, 5-androsten-3β-ol-17-one (DHEA), 5α-androstane-3β,17β-diol, and testosterone. The contamination of prohibited substances in other nutritional products has been reported elsewhere (19–21,27).

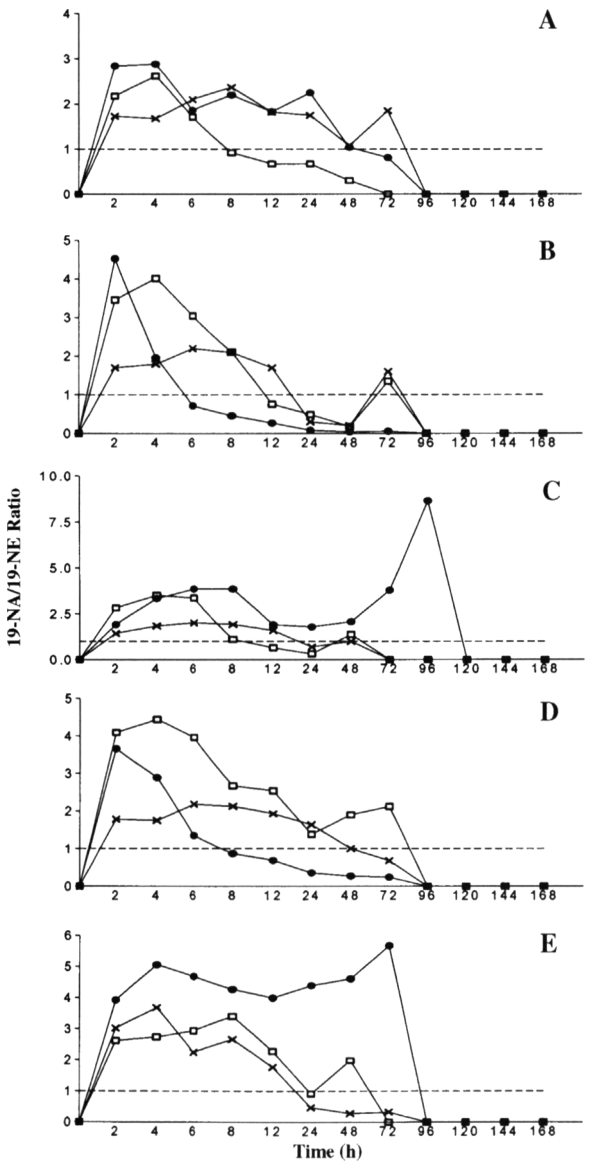

Figure 6. 19-NA/19-NE ratio profiles derived from excretion studies. (A) Nutritional supplement (n = 3); (B) nandrolone (n = 3); (C) 19-nor-4-androstene-3,17-dione (n = 3); (D) 19-nor-5-androstene-3β,17β-diol (n = 3); and (E) 19-nor-4-androstene-3β,17β-diol (n = 3).

Discrepancies were found between the amounts labeled in the supplement and that were detected and quantified by GC–MS. For example, as much as fivefold deviation was found in the amount of 5-androsten-3,17-dione between labeled (5 mg) and detected (25.5 mg). The amounts of the 10 identified anabolic steroids detected in one tablet ranged from 0.7 mg to 28.5 mg (see Table III).

In this study, five separate excretion studies were undertaken by oral administration of the following substances: (1) nutritional supplement (two tablets; n = 3); (2) authentic nandrolone (10 mg; n = 3); (3) 19-nor-4-androsten-3,17-dione (10 mg; n = 3); (4) 19-nor-4-androsten-3β,17β-diol (10 mg; n = 3); (5) 19-nor-5-androsten-3β,17β-diol (10 mg; n = 3). The results showed that 19-NA and 19-NE metabolites were present in urine from all the volunteers who administered the listed substances. These results confirmed the current findings that the administration of 19-norsteroids leads primarily to 19-NA and 19-NE metabolites (6,7,16). Although the derivation of metabolic products of 19-NA and 19-NE from the 19-norsteroids is still unclear, on the basis of various studies, Kohler and Lambert (9) indicated that 19-norsteroids are intermediates in the aromatization of androgens to estrogen. In one study, the yield of 19-NA and 19-NE metabolites was thought to be due to a quick conversion of 19-nor-4-androsten-3,17-dione to nandrolone in the body (21). It has been proposed that in the metabolic pathway, nandrolone metabolizes via oxidation of the 17β-hydroxy group into a keto group, and A-ring reduction results in the 5α and 5β isomers. The reduction of the 3-keto group, 3α-hydroxylation, is the dominant metabolic pathway in humans (19,24,25).

Androstenedione (4-androstene-3,17-dione), also listed as an ingredient in the supplement, has been found to metabolize into 19-NA in the urine samples of all subjects who received 100 or 300 mg androstenedione (20). However, this result was not in agreement with others' results, in which androstenedione was not found to yield 19-NA in urine (7,27). In our separate study, we found that androstenedione did not yield 19-NA after a 10-mg dose was administered. The discrepancy may be due to dose differences used. However, androstenedione, a precursor of testosterone, has been found to increase testosterone level and T/E ratio (27–29).

In one recent study, 19-nor-5-androsten-3,17-dione was found to metabolize into 19-NA and 19-NE, but the excreted amount of the latter was predominated over the former (18). The other three identified steroids, including 4-androsten-3β,17β-diol, 5-androsten-3β,17β-diol, and 5-androsten-3,17-dione, however, were not found to yield 19-NA and 19-NE metabolites in urine (19,24).

Three contaminated anabolic steroids found in the supplement, including testosterone, 5-androsten-3β-ol-17-one (DHEA) and 5α-androstane-3β,17β-diol (5α-dihydrotestosterone), are endogenous anabolic steroids and have not been reported to metabolize into 19-NA and 19-NE in urine. Two contaminated 19-norsteroids, 19-nor-4-androsten-3β,17β-diol and 19-nor-5-androsten-3β,17β-diol, however, were metabolized into 19-NA and 19 NE in urine. On the basis of this study

and others, the metabolites of 19-NA and 19-NE found in urine, obtained from the volunteers who consumed the nutritional supplement, were possibly derived from the mixture of the three 19-norsteroids, that is, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol.

The metabolic profiles of 19-NA and 19-NE in urine of the volunteers, who received a single dose of the nutritional supplement, authentic nandrolone standard, 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol, followed a similar time course. The concentrations of the metabolites, 19-NA and 19-NE, reached peaks between 2 and 6 h post-administration and diminished in approximately 3 to 4 days. 19-NA and 19-NE were reported to be detectable in urine 7 to 10 days after a single 50-mg oral dose of norandrostendione (6,21). Figure 9 summarized the anabolic steroids that metabolized to 19-NA and 19-NE.

In the metabolic pathway, the amount of 19-NA and 19-NE glucuronides of exogenous origin was found in ratios varying from 1:1 to almost 25:1 (16). In the present study, we found that the 19-NA concentrations in urine were generally higher than that in 19-NE (19-NA/19-NE ratio >1.0) especially during the early stages of urinary excretion, that is, before 6 h post-administration. After this period of time, the ratios of 19-NA and 19-NE fluctuated and even reversed (19-NA/19-NE ratio < 1.0). This pattern was present in all the substances tested (see Figure 7). When the cumulative amount of the metabolites was evaluated throughout the urine collecting periods, there were 2 cases out of 12 showed the amount of 19-NE greater than 19-NA; one case was when nandrolone was administered and the other when 19-nor-5-androsten-3β,17β-diol was consumed (Figure 8).

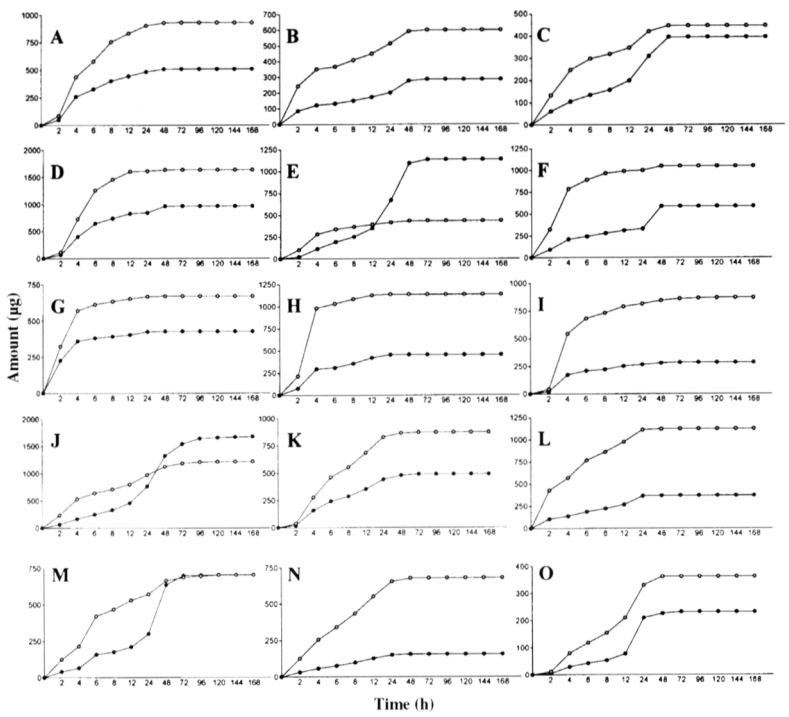

Figure 7. Cumulative amount of 19-NA (○) and 19-NE (●) excreted in urine after consumption of various substances: (A–C) nutritional supplement; (D–F) nandrolone; (G–I) 19-nor-4-androsten-3,17,-dione; (J–L)19-nor-5-androstene-3β,17β-diol; and (M–O)19-nor-4-androstene-3β,17β-diol.

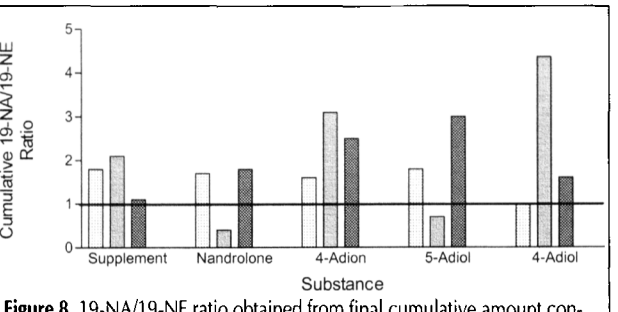

Figure 8. 19-NA/19-NE ratio obtained from final cumulative amount contained in urine of excretion studies (n = 3). 4-Adion denotes 19-nor-4-androstene-3,17-dione; 5-adiol denotes 19-nor-5-androstene-3β,17β-diol; and 4-adiol denotes 19-nor-4-androstene-3β,17β-diol.

In a recent study, the 19-norsteroids-derived metabolites, 19-NA and 19-NE, were found predominantly excreted in glucuro-conjugated form, which accounted for 65% to 93% of the total (16). In addition, around 30% of endogenous 19-NA was sulfo-conjugated, whereas 100% of 19-NA was glucuro-conjugated when nandrolone was administered (30). In the present study, there were two cases had the amount of 19-NE exceeded 19-NA. This result may be attributed to (1) individual metabolic differences; (2) differences in the amount of glucuro-conjugated and sulfo-conjugated metabolites produced; and (3) differences in substances administered (30). To this end, it would be interesting to look closely at differences in the conjugated moieties of the 19-NA and 19-NE metabolites with respect to various 19-norsteroids administered.

Conclusions

The present study demonstrated that the banned substances in sport were contaminated in the nutritional supplement tested. In addition, we showed that the administration of nutritional supplements, even in the absence of synthetic nandrolone, could yield the same metabolic products, 19-NA and 19-NE, as nandrolone. We confirmed that 19-nor-4-androsten-3,17-dione, 19-nor-4-androsten-3β,17β-diol, and 19-nor-5-androsten-3β,17β-diol could metabolize to 19-NA and 19-NE. Although the 19-NA/19-NE ratio is consistently greater than 1.0 during the early stages of metabolism, the ratio profiles may vary depending on the anabolic steroids consumed and individual variations in metabolism during the later stage of the excretion. The 19-NA and 19-NE metabolites in urine of the volunteers in this study were derived from a mixture of 19-norsteroids contained in the supplement. On the basis of these findings, we postulate that some of the nandrolone-related positive cases detected in doping control might

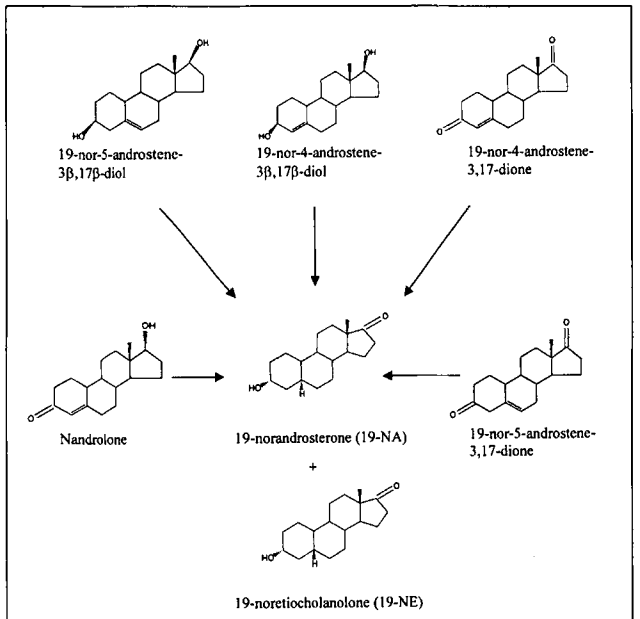

Figure 9. Sources of the anabolic steroids that metabolize into 19-NA and 19-NE.

have resulted from the consumption of nutritional supplements that contain 19-norsteroids.

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