

Trace Contamination of Over-the-Counter Androstenedione and Positive Urine Test Results for a Nandrolone Metabolite

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THE DIETARY SUPPLEMENT INDUSTRY generates annual sales estimated at US \$12 billion,¹ and in 1998 up to \$800 million of this was spent on "sports supplements."² An important consequence of the 1994 Dietary Supplement Health and Education Act³ is that several androgenic steroids, eg, androstenedione, dehydroepiandrosterone, 19-norandrostenedione, and 19-norandrostenediol, became widely available over-the-counter (OTC) in the United States and via the Internet. This act enables companies to market steroids as nutritional supplements if they do not claim to diagnose, prevent, or cure disease, in which case they escape US Food and Drug Administration regulation. Thus, content and purity of the ingredients are left to the manufacturer.⁴

Athletes take OTC steroids for their alleged muscle-building properties. Often the package labels do not warn that they may contain substances that are banned by sports organizations and may cause a positive urine test result.

Recently, there has been an increase in the number of athletes who

Context Several anabolic steroids are sold over-the-counter (OTC) in the United States, and their production is not regulated by the US Food and Drug Administration. Reports have suggested that use of these supplements can cause positive urine test results for metabolites of the prohibited steroid nandrolone.

Objectives To assess the content and purity of OTC androstenedione and to determine if androstenedione and 19-norandrostenedione administration causes positive urine test results for 19-norandrosterone, a nandrolone metabolite.

Design Randomized controlled trial of androstenedione, open-label trial of 19-norandrostenedione, and mass spectrometry of androstenedione preparations, conducted between October 1998 and April 2000.

Setting Outpatient facility of a university hospital.

Participants A total of 41 healthy men aged 20 to 44 years.

Intervention Participants were randomly assigned to receive oral androstenedione, 100 mg/d (n=13) or 300 mg/d (n=11) for 7 days, or no androstenedione (n=13); in addition, 4 patients received 10 µg of 19-norandrostenedione.

Main Outcome Measures Content of OTC androstenedione preparations; level of 19-norandrosterone in urine samples, determined by mass spectrometry, compared among the 3 randomized groups at day 1 and day 7, and among the participants who received 19-norandrostenedione from October 1998 to April 2000.

Results All urine samples from participants treated with androstenedione contained 19-norandrosterone, while no samples from the no-androstenedione group did. Urinary concentrations were averaged for day 1 vs day 7 measurements; mean (SD) 19-norandrosterone concentrations in the 100-mg/d and 300-mg/d groups were 3.8 (2.5) ng/mL and 10.2 (6.9) ng/mL, respectively ($P = .006$). The 19-norandrosterone content exceeded the cut-off for reporting positive cases (>2.0 ng/mL) in 20 of 24. The androstenedione preparation used was pure at a sensitivity of 0.1%, but at 0.001% 19-norandrostenedione was found. For the 4 participants to whom 10 µg of 19-norandrostenedione was administered, 19-norandrosterone was found in all urine samples. Of 7 brands of androstenedione analyzed at the 1% level, 1 contained no androstenedione, 1 contained 10 mg of testosterone, and 4 more contained 90% or less of the amount stated on the label.

Conclusion Our study suggests that trace contamination of androstenedione with 19-norandrostenedione is sufficient to cause urine test results positive for 19-norandrosterone, the standard marker for nandrolone use. Oral steroid doses as small as 10 µg are absorbed and excreted in urine. Some brands of androstenedione are grossly mislabeled. Careful analysis of androstenedione preparations is recommended in all studies of its biological effects.

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have tested positive for nandrolone, a banned steroid popular in the early years of athletic doping. Since nandro-

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lone itself is rarely found in urine, these cases are actually reports of findings of urinary 19-norandrosterone, the major but inactive metabolite of nandrolone (19-nortestosterone). Administration of nandrolone fell out of style in the 1980s because athletes know that it is readily detectable in urine for weeks.⁵ Our laboratory conducts approximately 15000 tests per year and reported 10, 23, and 35 cases where 19-norandrosterone was found in urine in 1997 to 1999, respectively.

19-Norandrosterone is also found in urine after ingestion of the OTC steroid 19-norandrostenedione.⁶ In sport, the origin of urinary 19-norandrosterone is moot because many sports organizations prohibit and test for OTC steroids in addition to controlled steroids. 19-Norandrosterone traces may occur naturally in the urine of untreated males^{7,8}; therefore, the International Olympic Committee set cutoffs in 1998 at 2 ng/mL for males and 5 ng/mL for females.

We recently reported that oral androstenedione administration increases serum testosterone and estradiol levels in young men.⁹ Because of reports suggesting that dietary supplement use can cause urine test results positive for 19-norandrosterone, we analyzed these men's urine samples for 19-norandrosterone. We also assessed the effect of administration of 10 µg of 19-norandrostenedione on urinary 19-norandrosterone excretion and examined the purity and content of commercial androstenedione preparations.

METHODS

Androstenedione Administration

The subjects (n=37, aged 20-40 years) and protocol have been described previously.⁹ The men were randomly assigned to receive no androstenedione (n=13) or 100 (n=13) or 300 mg/d (n=11) of androstenedione (Sports One, Klein Laboratories, Wallingford, Conn) for 7 days. Urine was collected for 8 hours on days 0 (baseline), 1, and 7. Urinary 19-norandrosterone concentrations were compared on days 1 and 7 and between the 100- and 300-mg groups by paired *t* tests. Data are ex-

pressed as the mean (SD), and $P < .05$ was considered statistically significant.

19-Norandrostenedione Administration

Four healthy men, aged 30 to 44 years, who denied use of anabolic steroids and steroid supplements, participated in the study after giving written informed consent. Each man collected urine before and after ingestion of 10 µg of 19-norandrostenedione (>99.0% pure). The UCLA Office for Protection of Research Subjects approved the study.

OTC Androstenediones, Standards, and Content

Over-the-counter capsules were purchased from stores. Androstenedione, testosterone, 19-norandrostenedione, and nandrolone were obtained from Sigma Chemical Company (St Louis, Mo). The capsules' androstenedione content was determined by high-performance liquid chromatography.

Impurity Analysis by Liquid Chromatography-Mass Spectrometry-Mass Spectrometry (LC-MS-MS)

Impurities in excess of 1% or 0.1% of the androstenedione were detected by LC-MS-MS. We analyzed 18 capsules from brands 1 and 2 for steroid contaminants at more than 0.1% of androstenedione and selected Sports One (brand 1) for the androstenedione clinical study.⁹ We also analyzed 26 capsules from brands 3 through 9 for androstenedione content and traces of steroids (>1% of the androstenedione).

Reanalysis for Impurities at 0.001%

We reanalyzed 8 capsules from 4 bottles of brand 1 by LC-MS-MS for 19-norandrostenedione or nandrolone impurities at more than 0.001% of the androstenedione.

Analysis of Urine Samples for 19-Norandrosterone and 19-Noretiocholanolone

Steroid extraction and derivatization, with minor modifications, have been

previously described.¹⁰ The analysis used a high-resolution mass spectrometer. Full scans of 19-norandrosterone were obtained on 10 samples with 19-norandrosterone concentrations greater than 5 ng/mL.

RESULTS

Initial Androstenedione Capsule Analysis

We analyzed 18 capsules from brands 1 and 2 to select a brand for the androstenedione clinical study. This analysis detected impurities at greater than 0.1% of androstenedione. Both brands were more than 99.9% pure, and brand 1 was selected (TABLE 1).

Survey of Commercial Androstenediones

We also analyzed 26 capsules from brands 3 through 9 at a less stringent level (1%) of impurity. Table 1 shows that brand 8 contained no steroids and brand 9 contained 10 mg of testosterone. The mean androstenedione content of the brands labeled as containing 100 mg varied from 85 to 103 mg. The amounts of androstenedione in brands 1, 3, and 4 were within ±10% of that stated on the label, whereas brands 2 and 5 through 9 contained less than 90%.

Androstenedione Administration Study

19-Norandrosterone was found in the urine samples of all subjects (n=24; range, 1.3-23.1 ng/mL) who received androstenedione, in none of the baseline urine samples (n=37), and in none of the day 1 (n=13) or day 7 urine samples (n=13) from the 0-mg/d group (TABLE 2). There was no difference in 19-norandrosterone concentrations between day 1 and day 7 ($P = .06$ and $P = .67$ for the 100 mg/d and 300 mg/d groups, respectively); thus, the mean concentrations of day 1 and day 7 were averaged. The mean 19-norandrosterone concentrations in the 100- and 300-mg/d groups were 3.8 (2.5) ng/mL and 10.2 (6.9) ng/mL, respectively. These concentrations differed ($P = .006$), indicating dose dependency (Table 2).

Reanalysis of Brand 1

After finding significant amounts of 19-norandrosterone in the urine samples from men who received brand 1, 2 capsules from each of 4 bottles of brand 1 androstenedione were reanalyzed using a method 100 times more sensitive to detect 19-norsteroids if present at a level greater than 0.001% of the androstenedione. None of the capsules contained nandrolone or 19-norandrostenediol; however, 7 of 8 contained 0.004% to 0.018% 19-norandrostene-

dione (mean, 0.0076% or 7.6 µg per capsule).

19-Norandrostenedione Administration Study

The urine samples of all 4 subjects who ingested 10 µg of 19-norandrostenedione contained both 19-norandrosterone and 19-noretiocholanolone (TABLE 3), neither of which was found in the baseline urine samples. The 8-hour recovery of 19-norandrostenedione, measured as equivalents of 19-

norandrosterone and 19-noretiocholanolone, was 48% (range, 32%-66%) of the total 10-µg dose.

COMMENT

We found 19-norandrosterone, a metabolite of the prohibited steroid nandrolone, in all urine samples obtained after oral administration of androstenedione and in none of the urine samples collected before androstenedione administration. The mean concentrations were small. Nevertheless, if these samples were from athletes, 20 of 24 men would have tested positive for 19-norandrosterone (International Olympic Committee cutoff, 2 ng/mL for males). Thus, the administration of androstenedione contaminated with 19-norandrostenedione results in 19-norandrosterone in the urine, as does the administration of nandrolone. However, the ingestion of 19-norandrostenedione as a trace impurity in androstenedione is readily distinguished from that of nandrolone use because the latter results in very high urine concentrations of 19-norandrosterone (>100 000 ng/mL),⁶ whereas in this androstenedione study the highest mean concentration was only 23.1 ng/mL.

Brand 1 was selected for the clinical study because it had the most consistent content of androstenedione (Table 1) and less than 0.1% impurities. After the unexpected finding of 19-norandrosterone in urine, we retested brand 1 for steroid traces if present at a level greater than 0.001%, which could explain this finding. Only brand 1 was retested; thus, we have no data on trace contamination of other androstenedione preparations.

The 19-norandrosterone detected in the androstenedione subjects' urine samples could result from metabolism of the administered androstenedione to 19-norandrosterone, stimulation of a latent metabolic pathway to produce endogenous 19-norandrosterone, or oral administration of the 19-norandrostenedione impurity in androstenedione. We cannot exclude metabolism of androstenedione to 19-norandrosterone. Aromatizable androgens such

Table 1. Summary of the Purity and Content of Capsules of Over-the-Counter Androstenediones*

Brand No.	No. of Capsules Tested	Steroid and Dose Listed on Label†	Steroids Found	Mean Amount, mg
1‡	13	Androstenedione, 100 mg	Androstenedione	93.1§
2	5	Androstenedione, 100 mg	Androstenedione	82.8
3	4	Androstenedione, 100 mg	Androstenedione	103
4	2	Androstenedione, 100 mg	Androstenedione	90
5	4	Androstenedione, 100 mg	Androstenedione	88
6	4	Androstenedione, 100 mg	Androstenedione	85
7	4	4-Androstene 3, 17-dione, 50 mg¶	4-Androstene 3, 17-dione	35
8	4	Androstenedione, 50 mg	None	0
9	4	4-Androstene 3, 17-dione, 250 mg¶	4-Androstene 3, 17-dione	168
		(not listed on label)	Testosterone	10

*Brands 1 and 2 and 3 through 9 tested for impurities at 0.1% and 1%, respectively.

†Exactly as listed on the label.

‡Brand used for our study in reference 9.

§Range, 84-107 mg.

||Range, 76-84 mg.

¶4-Androstene 3, 17-dione = androstenedione.

Table 2. Number of Subjects Categorized by Concentration and Mean Concentration of 19-Norandrosterone in Urine Samples Collected 0 to 8 Hours After Administration of 100 or 300 mg of Androstenedione*

Dose, mg	No. of Subjects					Mean (SD) Concentration, ng/mL
	<0.5 ng/mL	0.5-2.0 ng/mL	2.1-5.0 ng/mL	5.1-10 ng/mL	11-35 ng/mL	
0	13	0	0	0	0	0
100	0	3	7	3	0	3.8 (2.5)†
300	0	1	1	5	4	10.2 (6.9)

*Sports test results were considered negative when concentrations were less than or equal to the 2.0 ng/mL cutoff and positive when concentrations were greater than 2.0 ng/mL.

†Significantly different from the 300-mg dose ($P = .006$).

Table 3. Mean Concentrations of 19-Norandrosterone and 19-Noretiocholanolone After Oral Administration of 10 µg of 19-Norandrostenedione to 4 Subjects

Time, h	Concentration, Mean (Range), ng/mL	
	19-Norandrosterone	19-Noretiocholanolone
0-2	17.2 (2.8-48.9)	5.2 (1.1-16.7)
3-4	5.6 (4.4-7.6)	1.3 (0.6-1.9)
5-6	1.2 (0.6-1.7)	0.3 (0.1-0.3)
7-8	0.5 (0.1-0.8)	0.2 (0.1-0.3)

as androstenedione are readily converted to estrogens in humans, and this conversion is associated with C-19 demethylation to 19-norsteroids.¹¹

We cannot exclude the possibility of stimulation of a latent pathway either. There are reports of up to 0.6 ng/mL of 19-norandrostosterone in the urine of untreated males,^{7,8} of 19-nortestosterone and 19-norandrostenedione in human ovarian follicles,¹² and of 19-nortestosterone in the serum of pregnant women.¹³ However, none of the 37 subjects who received androstenedione or the 4 subjects who received 19-norandrostenedione had 19-norandrostosterone levels greater than 0.5 ng/mL in their baseline urine samples. These 41 cases together with the existing 47 cases^{7,8,14} add support to the suggestion that the upper limit of endogenous 19-norandrostosterone production does not result in urine concentrations of 19-norandrostosterone greater than 0.6 ng/mL.

The hypothesis that the 19-norandrostosterone is derived from the 19-norandrostenedione contamination is strongly supported by the finding that administration of 19-norandrostenedione produced urinary concentrations of 48.9 ng/mL of 19-norandrostosterone and 16.7 ng/mL of

19-noretiocholanolone in the first 2 hours and a 48% recovery of the 19-norandrostenedione in urine as the metabolites 19-norandrostosterone and 19-noretiocholanolone. Therefore, the 19-norandrostosterone found in this study is almost certainly due to ingestion of contaminated androstenedione.

The manufacturer of the particular batch of brand 1 androstenedione that we reanalyzed should not be faulted for a product with a maximum contamination of 0.018%. This level of contamination is far below the level of 0.1% accepted by the Food and Drug Administration for pharmaceuticals.¹⁵ In contrast, some of the other products contained less drug than was listed on the label and even misrepresented ingredients. According to the Dietary Supplement Health and Education Act,³ both brand 8 (no androstenedione) and brand 9 (fails to identify testosterone) are misbranded. If we apply the *United States Pharmacopoeia*¹⁶ criterion of $\pm 10\%$ for ethical pharmaceuticals, then brands 2, 5, 6, and 7 are misbranded (wrong strength). Thus, 6 of 9 brands tested were misbranded.

Today most steroids are made from plant precursors, eg, diosgenin from yams and stigmasterol from soya, by

semisynthesis¹⁷ or bacterial action.¹⁸ These publications do not provide sufficient detail to determine how the contamination of androstenedione with 19-norandrostenedione might have occurred. It is also possible that the contamination occurred after synthesis, for example, during capsulation or some other packaging or formulation step.

Sport organizations that test for anabolic steroids have banned androstenedione even though it is sold OTC in the United States. The data reported herein show that contaminated androstenedione administration will, like nandrolone and 19-norandrostenedione, be detected as 19-norandrostosterone in urine and can produce a positive test result for anabolic steroids in subjects taking OTC dietary supplements.

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