Effect on Sports Drug Tests of Ingesting Meat from Steroid (Methenolone)-Treated Livestock

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Anabolic-androgenic steroids are widely misused in human sports and are also used as growth promoters in livestock. Athletes who consume meat containing such hormone residues may risk failing a sports drug test. Prompted by an athlete's defense case, we questioned whether the consumption of small livestock given doses of anabolic steroid, orally or intramuscularly, could generate positive results in samples tested by our analytical procedures. We analyzed urine from eight men who consumed chickens that had been either fed with methenolone acetate (1 mg/day) from day 0 to 21 or injected with methenolone heptanoate depot (1 mg/intramuscular injection) on days 0, 7, and 14 and slaughtered on day 22. No methenolone or characteristic major metabolite was detected in samples from subjects who ate meat from the orally dosed chickens. However, 50% of the samples collected 24 h after consumption of the intramuscularly dosed chickens were confirmed positive. Hence, eating meat containing small amounts of injected hormone may constitute a serious liability to the athlete.

Indexing Terms: *sports medicine/anabolic steroids/abused drugs/dietary effects*

For drug testing in sports, the misuse of anabolic-androgenic steroids continues to account for the largest proportion of positive cases reported by the International Olympic Committee (IOC) Accredited Laboratories.[5] These hormones are also used for the promotion of weight gain and feed efficiency in livestock such as cattle, sheep, and, to a much lesser extent, poultry (1). In some countries the use of veterinary growth promoters are restricted: e.g., in the US, the anabolic-androgenic steroids exempted by the Federal Controlled Substances Act include the esters of testosterone and trenbolone that are administered as solid implants in the ear; after slaughter, the ear is discarded to prevent accidental ingestion. In other countries, such as within the European Economic Community (2), the use of anabolic agents for such purposes is now totally prohibited. Total prohibition rather than restricted use can cause a much greater incidence of illegal use, despite regulatory residue analysis, with the extent of misuse varying between countries. For example, evidence suggests that in Belgium one has a high risk of eating contaminated meat originating from large animals because of the presence of hormone residues in low-grade minced tissues, such as tail base or neck muscle (3,4). Such tissues can be the site of a covert hormone depot, usually administered in liquid form, that sometimes can remain undetected in meat after slaughter. Consequently, athletes who have eaten such meat may fail a drug test, their risk increasing with repeated consumption.

This collaborative study was initiated after the Norwegian IOC Accredited Laboratory confirmed the presence of a metabolite of a banned steroid in the urine of a top-level athlete, who claimed in his defense that he had eaten a contaminated chicken. The compound identified was 3α-hydroxy-1-methylen-5α-androstan-17-one, the major urinary metabolite of methenolone (17β-hydroxy-1-methyl-5α-androst-1-en-3-one), which is excreted mainly as the glucuronide conjugate (5,6). Methenolone is orally active but is also obtainable in an oily solution for injection. The presence of methenolone (Fig. 1A, inset) and (or) its major metabolite (Fig. 1B, inset) in athletes' samples contributes significantly to the large number of positives internationally in the class of anabolic agents. Whereas eating contaminated meat from large species can give a positive test result so that the subject fails a doping test, we questioned whether the same could be true from eating poultry, in which the total amount of steroid administered would be much smaller. In particular, the study was designed to test the hypothesis that only livestock given depot injections into edible muscle sites, as opposed to steroid in the feed, could result in such significantly contaminated meat.

To investigate these possibilities, we collected urine from volunteers who ate chickens that had received methenolone, either as the acetate in their feed or intramuscularly as the heptanoate ester. The samples were analyzed by our routine screen for urinary anabolic steroids, which is designed to detect all the anabolic steroids known to be misused in sport. The screen incorporates selected-ion monitoring gas chromatography–mass spectrometry (GC-MS), with a positive result by this screen rendering the sample "suspect." In keeping with our standard operating procedures, all suspect samples were then subjected to confirmatory analysis by full-scan GC-MS.

For comparative purposes, the same volunteers ingested a selected dose of methenolone as the acetate and heptanoate ester, and their urine samples were analyzed to investigate the incidence of positives by our screening procedures.
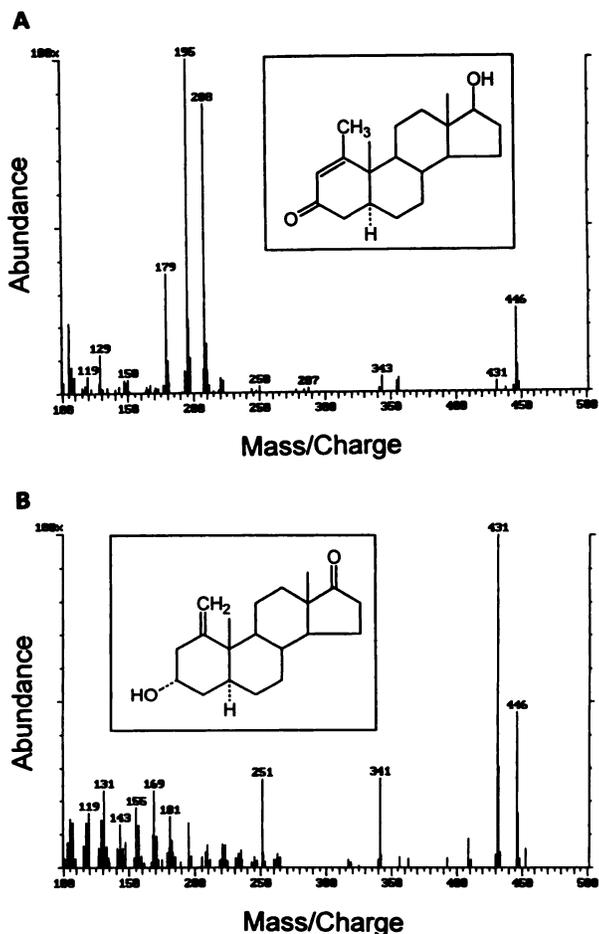
Fig. 1. Background-subtracted full-scan mass spectra of the bis-TMS ether derivatives of a urinary extract containing (*A*) methenolone and (*B*) its major metabolite, 3α-hydroxy-1-methylen-5α-androstan-17-one.

The urine was collected from a volunteer who had eaten meat contaminated with methenolone. The *insets* show the respective chemical structures (underivatized).

Materials and Methods

Hormone Administration to Chickens

On day 0, 30 chickens, each 2 weeks old, were randomly divided into three groups of 10, designated as the control, oral, and intramuscular groups. The control group received no steroid administration. The oral group received 1 mg/day of methenolone acetate (Primobolan) mixed in with their feed from day 0 to day 21. The intramuscular group received 1-mg injections of methenolone heptanoate (Primobolan depot) into their thigh muscles on days 0, 7, and 14. On day 22 all the chickens were slaughtered and dressed for consumption. The mean weight of the dressed chickens for the control, oral, and intramuscular groups were 813, 853, and 863 g, respectively. The chickens were stored frozen (−18°C) until the evening before cooking, when they were left to thaw at 4°C.

Volunteers' Consumption of Chicken and of Steroids

The procedures followed were in accordance with the ethical standards of our institutions and informed consent was obtained from all the subjects. The eight

healthy and very fit male subjects (ages 30–55 years; weight range 72–90 kg) who volunteered for this study were considered normal according to body-mass-for-height index for their body frame size (7).

Each volunteer was given a whole roasted chicken to eat at noon on days 0, 1, and 2. Chickens from the control group were given on day 0, from the oral group on day 1, and from the intramuscular group on day 2. The volunteers were not informed of the sequence nor of any randomization procedure. All volunteers emptied their bladders just before each meal, then collected urine samples at 4–6 h and 22–24 h afterwards. Additionally, after eating the intramuscularly treated chickens, they collected another urine sample between 46 and 48 h.

Five months later, the same volunteers ingested 0.1 mg of methenolone acetate at noon on day 1 and 1 mg of methenolone heptanoate on day 3 with a normal meal. Urine samples were collected as described above.

Urine Analysis

Screening procedure. Our routine screening procedure for anabolic steroids incorporates enzyme hydrolysis, extraction, and derivatization followed by selected-ion monitoring GC-MS incorporating a mass selective detector (Model 5890/5970B; Hewlett-Packard, Berks, UK). The full methodology followed, including the column used and temperature conditions, is as described for the analysis of testosterone and epitestosterone (8). In keeping with our standard operating procedures, the system was adjusted to ensure that the retention time of the bis-trimethylsilyl (bis-TMS) ether derivative of the internal standard, 16,16,17-trideuterated testosterone (m/z 435), was 13.1 ± 0.1 min. For detection of methenolone administration, four ions are monitored; the bis-TMS ether derivative of methenolone (m/z 208 and 195) and the major metabolite (m/z 446 and 431). To avoid the possibility of interfering components giving inaccurate relative abundance ratios, we do not use abundance ratio criteria as qualifiers for the screen. Instead, a sample fails the screen for methenolone or metabolite, i.e., is a "suspect" positive, if all four ions are maximized at a retention time within 0.1 min of that obtained for the reference standards (typically 13.7 min for methenolone and 11.8 min for metabolite; see below).

Confirmatory procedure. The presence of methenolone and (or) the major metabolite was confirmed by electron impact full-scan analysis with an ion-trap detector (ITD 800; Finnigan MAT, Herts, UK) coupled to a gas chromatograph (Model 5890A; Hewlett-Packard). In our experience (unpublished), the use of the ion trap gives greater sensitivity than the use of our mass-selective detectors in full-scan mode. Apart from the use of an ion-trap mass spectrometer, our confirmatory procedure is essentially the same as described for the screening procedure with three modifications:

1) The steroid glucuronide conjugates are rapidly hydrolyzed at 50°C with glucuronidase from *Escherichia coli* (Sigma Chemical Co., Dorset, UK; 2500 Fishman Units in 500 μL of acetate buffer) instead of *Helix poma-*

tia. The advantage of using this enzyme is that it is considered not to have any 17β-hydroxysteroid dehydrogenase activity, which is an undesirable and significant risk of using uncharacterized batches of enzyme from *H. pomatia (9,10)*. The disadvantages are that the *E. coli* enzyme has no sulfatase activity and is more expensive. Hence, in our laboratories, we reserve its use for confirmatory procedures as necessary for unequivocal identification of a banned substance.

2) We use hexane (5 mL) instead of diethyl ether for extraction, which reduces the amount of interfering components that can coelute with methenolone and the major metabolite.

3) The conditions for the ion trap GC-MS are identical to those described for screening analysis, e.g., temperature program and column used *(8)*, and hence the retention time criteria are the same. However, if interfering ions arise in the full-scan spectra for methenolone, the derivatized samples are reinjected and a slower oven temperature program is used, i.e., 180°C for 1 min, followed by a 2°C/min increase to 250°C and a 5°C/min increase to 280°C. This temperature program is suitable for good chromatographic separation of methenolone from these interfering components, giving retention times for the bis-TMS ether derivative of methenolone, metabolite, and 16,16,17-trideuterated testosterone of 24.1 ± 0.1, 20.4 ± 0.1, and 23.2 ± 0.1 min, respectively.

Full-scan spectra of the bis-TMS ether derivatives were compared with those of authentic reference standards of methenolone (Schering, Berlin, Germany) and its major metabolite (synthesized by W. Schänzer, Deutsche Sporthochschule, Cologne, Germany). The mass spectrum for the bis-TMS ether derivative of methenolone includes the presence of the molecular ion m/z 446 (M$^+$) and the daughter ions m/z 208, 195, and 179. Fig. 1A shows a typical spectrum for a urine sample from the volunteers who had ingested meat containing methenolone (see *Results*). The characteristic mass spectrum for the bis-TMS ether derivative of the major metabolite eliminated into urine incorporates the molecular ion m/z 446 (M$^+$) and the daughter ions m/z 431 (M − 15)$^+$, 341 (M − 15 − TMSOH)$^+$, 251 (M − 15 − bis-TMSOH)$^+$ (Fig. 1B). Criteria for confirmation of samples required concordance in retention times (±0.3%) and mass spectra with that of the reference standards. In general, for positive confirmation of methenolone, no ion of m/z >208 and <446 was to be present in abundance greater than that of m/z 446. Similarly, for the metabolite, no ion of m/z >251 and <446 (apart from m/z 431 and 341) was to be present in greater abundance than that of m/z 251. Finally, all of the characteristic ions were to be at least threefold the background noise for m/z ranges of 150–550 for methenolone and 250–550 for its major metabolite.

Results

After eating the chickens from the control or orally treated group, none of the subjects gave urine samples (4–6 h and 22–24 h postmeal) that were positive in the screen. These results contrast with the large number of positives detected after eating the chickens from the intramuscularly treated group, as tested by both our screening and confirmatory procedures (Table 1). Confirmatory analysis of these samples unequivocally identified the presence of methenolone much more so than its major metabolite. The mass spectra obtained greatly exceeded our general requirements for the absence of interfering ions in the specified m/z ranges described (for methenolone and its metabolite, no interfering ion was >30% of the abundance of m/z 446 and 251, respectively). In addition, full-scan data suggested the presence of metabolite in the other six samples for the 4–6 h period, three samples for the 22–24 h period, and two samples for the 46–48 h period. In these cases, the mass spectra obtained were characteristic of the metabolite, but unequivocal identification was not possible because of one or two interfering ions, which violated our criteria. Hence, these samples were reported negative for presence of metabolite. We did not consider it necessary to undertake extensive work to achieve better spectra because these samples were already confirmed positive for methenolone.

After the volunteers ingested 0.1 mg of methenolone acetate, none of their urine samples were positive, such a dose being too small to be detected. However, after oral administration of 1 mg of methenolone heptanoate, the number of their urine specimens that were positive by our screening procedure were: all the samples from the 4–6 h period, 7 of 8 from the 22–24 h period, and 6 of 8 from the 46–48 h period; the mean concentration of metabolite in these samples was estimated (based on m/z 431) as 25, 10, and <5 μg/L, respectively.

Discussion

This study supports the hypothesis that meat originating from animals fed with orally active anabolic-androgenic steroids contains too little residue for consumption to cause an athlete to fail the current drug tests. Conversely, eating meat contaminated with depot injection sites can result in unequivocal identification of banned substances in urine. Hence, an acute dose of hormone from contaminated meat constitutes a serious liability to the athlete.

For cattle, steroid doses can be as great as ~1 mg/kg body weight; we used a similar amount for our study

Table 1. Results for urine samples collected from volunteers (n = 8) after eating chickens treated with intramuscular methenolone heptanoate.

Time post meal, h	No. of positive results		
		Confirmation[b]	
	Screen[a]	Parent	Metabolite
4–6	8	7	2
22–24	8	4	0
46–48	4	1	0

[a] Selected-ion monitoring GC-MS.
[b] Full-scan GC-MS.

with chickens. In humans, the total urinary excretion of both methenolone and its major metabolite is <5% of the ingested dose, with a large proportion of the parent steroid being rapidly eliminated into the urine between 1 and 12 h after ingestion (11). However, with our sensitive methods, even the consumption of relatively small amounts of residual methenolone from injected chickens meant that enough was eliminated 24 h later to enable positive confirmation of the steroid in four of the eight volunteers.

Addition of the heptanoate ester to methenolone decreases the polarity of the steroid and hence its rate of release from the oily injection vehicle into the systemic circulation for activation by esterases. Such depot activity accounts for residual drug in the chicken muscle at the time of slaughter, even at 8 days after the last injection. Humans ingesting the contaminated meat, or taking an oral administration per se, apparently have rapid intestinal absorption of the heptanoate ester of methenolone, and the parent steroid is detected in the urine shortly afterwards.

Compared with the recommended therapeutic dose of methenolone (e.g., methenolone acetate 10 to 20 mg/day), a small dose of depot hormone from eating contaminated meat is unlikely to cause a significant physiological effect. However, one should also duly consider the possibility of endocrine disorders arising from chronic consumption, especially in women and children. In sport, eating such meat constitutes a serious liability to the athlete undergoing a drug test. However, the tribunal hearing the case of the Norwegian athlete was not convinced by the claim that he had eaten contaminated meat, and he lost his appeal.

Analytically, no currently accepted method is capable of distinguishing between a sample from a cheater and one from an athlete who has eaten contaminated meat. Debruyckere et al. (3) suggest that repeated sampling will differentiate between a chronic misuser and the athlete found positive due to inadvertent consumption, because the latter will test negative a few days later. However, some chronic misusers who anticipate testing switch to orally active anabolic steroids and stop at some time before an event in an attempt to test negative. In such cases, the amounts of steroid or metabolite eliminated upon sampling may be very similar to that from ingestion of hormone residues, such that both the innocent athlete and the cheater could test positive at that time and samples collected thereafter would test negative. Hence, for the athlete found positive due to inadvertent consumption, successful litigation against the meat supplier may be the best avenue of defense. Such a strategy could also be followed in countries permitting the administration of selected veterinary anabolic-androgenic steroids but where the residue amounts tolerated have been exceeded. Overall, the continuous use of stringent legislative measures pertaining to the use of growth promoters in animals is probably the best safeguard against the potential hazard to the athlete and society at large.

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