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Illegal Use of Anabolic Agents in Animal Fattening - Consequences for Doping Analysis

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Introduction

In Europe the illegal use of anabolic agents in animal fattening started with diethylstilbestrol at least 15 - 20 years ago[1]. Due to the development of efficient control procedures it changed to other estrogenic and androgenic compounds as e.g. estradiol, zeranol, nandrolone and testosterone in the 1980s and since 1988 the steroids have been supplemented by β-agonists like clenbuterol and salbutamol[2]. Already since 1981 the use of stilbenes was prohibited and since 1988 the use of substances having hormonal actions in livestock farming is forbidden in general in the European Union[3].

Although the European Union has developed a more and more effective control system[4,5] the illegal use of anabolic agents in animal fattening has created considerable concern and discussions related to the consequences of the consumption of contaminated meat. These consequences range from health risks[6] to positive results in doping analysis in sports[7].

The overlap of the compounds on the IOC doping list[8] and the compounds banned for use in livestock farming consists mainly of the anabolic agents as anabolic androgenic steroids and beta-2-agonists[9].

When discussing the probability of testing positive in a doping control after having consumed contaminated meat, the concentrations in animal tissue after application of anabolic agents are of interest. Many reports on residue analysis in different tissues are available[10,11] but the very small probability of having an injection site processed in a meal makes the evaluation

difficult. In order to enlighten this problem to some extent we performed a controlled study with voluntary men that ate meat from cattle treated with anabolic agents.

Materials and methods

Study design

Four young cattles were treated with intramuscularly injected nandrolone or orally administered clenbuterol. Doses, duration and withdrawal periods (time since administration of last drug dose) before slaughtering are listed in Tables 1 and 2.

	Dose	Frequency	Total no. injections	Withdrawal period
Bull 1	1 mg/kg	every 4. week	3	28 days
Bull 2	1 mg/kg	every 4. week	3	61 days

Table 1 Administration of nandrolone decanoate i.m. to two bulls with an initial body weight of 314 and 341 kg, respectively.

	Dose	Frequency	Administration period	Withdrawal period
Calf 1	2 x 5 g/kg	daily	43 days	1 day
Calf 2	2 x 5 g/kg	daily	37 days	6 days

Table 2 Administration of clenbuterol orally to two calves with an initial body weight of 40 and 45 kg, respectively.

Experiment	Meat from	Withdrawal period
1	Calf 1	1 day
2	Calf 2	6 days
3	Bull 1	61 days injection site
4	Bull 2	28 days injection site
5	Bull 1	61 days
6	Bull 2	28 days
7	non treated animal	-

Table 3 Consumption of meat from treated and untreated animals.

The injection site from the nandrolone ester administration were labelled carefully. After slaughtering ca. 3 kg of meat were carved out of the thighs of the animals. For the bulls the injection sites were included twelve male volunteers (40 ± 9 years, 85 ± 9 kg) consumed seven meals (experiment 1-7) consisting of 300 g of beef meat (Table 3). Urine samples were collected from the participating men just before and 2, 4, 6, 10, 22 and 46 hours after meat consumption.

Sample preparation and derivatisation

A) Sample preparation for the routine analysis of norandrosterone and clenbuterol was done according to our routine procedure for the combined and free steroid fraction, respectively[12], including the derivatisation with MSTFA/NH_4I/Dithioeritritol (500:1:2).

B) To get a cleaner extract for clenbuterol analysis a solid phase extraction with Bond-Elut Certify (Varian, Harbour City, USA) was included before derivatisation.

Instruments

A) Hewlett-Packard GC-MSD 5890/5970, fused silica capillary column, crosslinked methyl silicone (Ultra 1), 12m, i.d. 0.2 mm, 0.11 μm film thickness. Selected ion monitoring detection, dwell time 50 ms.
Our identification limits for norandrosterone and clenbuterol are 2 and 1 mg/ml, respectively.

B) Hewlett-Packard GC 5890/Finnigan MAT 95 HRMS, column as in instrument A), multiple ion detection at resolution R=3000.
Our limit of quantitayion for clenbuterol is 10 pg/ml.

Results

All urine samples collected before and between 4 and 6 hours after the meals in experiment 1-7 were analysed according to our routine analysis methods (A). The laboratory staff were blinded with respect to sample coding.
In none of the analysed samples norandrosterone or clenbuterol were identified. Nevertheless six of the samples showed unregularities in the screening window for clenbuterol. After

breaking the sample codes it was shown that all these samples belonged to the 4-6 hours urine of experiment 1. The consumed meat in this experiment was from the calf treated with clenbuterol until one day before slaughtering.
All the samples from experiment 1, urine collection 4-6 hours, were reanalysed using an improved sample preparation and a more sensitive and specific detection (B). The results of the clenbuterol quantification is shown in Table 4, together with the clenbuterol concentrations of all urine samples from experiment 1 for four volunteers. The concentrations are plotted against the time after meat consumption in Figure 1.

Sample code	1 0 h	2 0-2 h	3 2-4 h	4 4-6 h	5 6-10 h	6 10-22 h	7 22-46 h
PB-1	x	x	x	120	x	x	x
TA-2	x	x	x	200	x	x	x
SH-3	x	x	x	160	x	x	x
OED-4	x	x	x		x	x	x
HH-5	x	x	x	220	x	x	x
SN-6	0	80	240	450	120	270	120
MB-7	0	40	140	370	850	160	100
VN-8	0	30	60	340	310	290	140
GM-9	x	x	x	160	x	x	x
FH-10	x	x	x		x	x	x
PD-11	x	x	x	250	x	x	x
OAD-12	0	30	50	140	90	110	160

Table 4 Quantification of clenbuterol (pg/ml) in urine samples collected after consumption of meat from a calf which was treated with clenbuterol until 1 day before slaughtering. Samples not analysed are indicated with x and the time period of urine sampling after the meal is indicated in hours (h).

Discussion

The estimation of the probability of finding positive doping control samples after ingestion of contaminated meat will always be speculative to some extent. The results of our study show that it is unlikely to find positive findings after administration of a "normal" dose of nandrolone decanoate and a withdrawal time of 4 weeks before slaughtering. This is half the

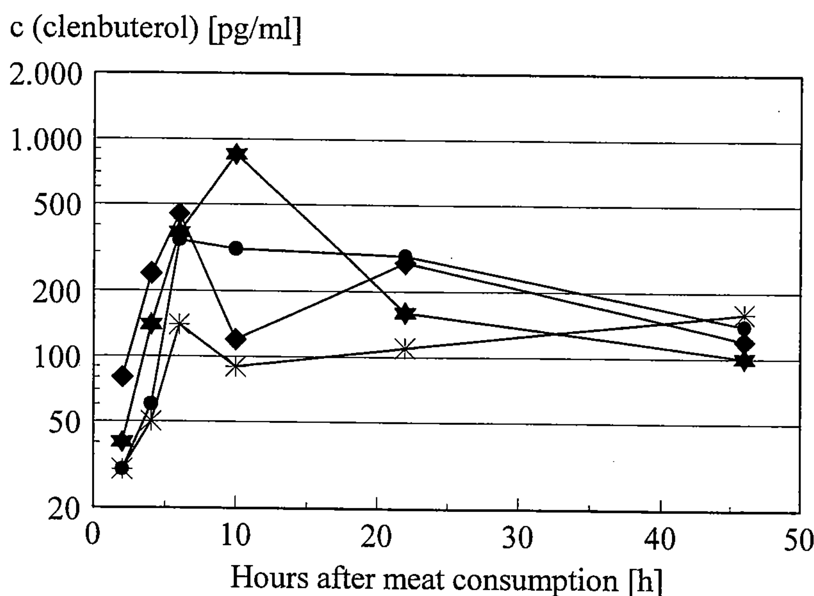

Figure 1 Quantification of clenbuterol in urine from 4 study subjects after consuming contaminated meat.

time which is allowed in the United States where administration is still legal. Of course we never can be sure about the real withdrawal times in illegal practices, but it is worth to remind at this point that in any case the residue levels of first-quality meat as steak are much too low to interfere with urinary doping control[7] . By choosing a cut-off limit of 2ng/ml norandrosterone also the "positive" findings of Debruyckere et al. after consumption of minced beef meat in Belgium[7] would drop below this concentration between 4 and 7 hours after the meal.

Concerning clenbuterol our study design includes two experiments with a withdrawal time of 6 and 1 days, respectively. The withdrawal time of one day is so short that it seems to represent the "worst" case. Even in this case we did not find positive findings when using the routine analysis with a limit of identification of 1 ng/ml urine.

The concentrations of clenbuterol in urine samples of experiment 1 obtained with a more sensitive method ranged from 120 to 450 pg/ml in the 4-6 hours urine collection, while one study object reached 850 pg/ml between 6 and 10 hours. Taking into account the concentrations of clenbuterol in muscle tissue after administration of clenbuterol to calves found by Meyer et al.[11] , which are in the low ng/g range (3.4-4.6) , a 300 g meal would resemble an intake of 1.2 µg clenbuterol. The normal therapeutic dose of clenbuterol lies 10-20 times higher and the peak concentrations of clenbuterol after one dose excretion studies

are ca. 10 times higher than the concentrations in experiment 1[13]. In this connection it is of interest that other tissues , f. ex. liver, accumulate clenbuterol to a much higher extent (ca. 10 times)[11] and that concentrations of clenbuterol after consumption of contaminated liver might be expected at the therapeutic level[6].

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